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Silicate-Solubilizing Bacteria in Louisiana Soils: Identification, Profiling, and Functions in Crop Production

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SILICATE-SOLUBILIZING BACTERIA IN LOUISIANA SOILS: IDENTIFICATION, PROFILING, AND FUNCTIONS IN CROP PRODUCTION

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Abstract

Studies were conducted to determine the potential of silicate-solubilizing bacteria (SSB) as biostimulant in Louisiana rice (*Oryza sativa* L.) production systems. Isolation and profiling of SSB in Louisiana soils; evaluation on its effects on the silicon (Si) uptake and productivity of rice using various carriers derived from slag, rice hull and sugarcane (*Officinarum* spp.) bagasse; and development of a feasible approach of incorporating SSB to the rice production system were conducted. Results showed that numerous bacteria isolated from Louisiana soils can solubilize silicate and produce multiple plant growth-promoting compounds. These potential SSB were identified into four genera: *Aeromonas, Bacillus, Enterobacter* and *Pseudomonas*. In the greenhouse, the differences in agronomic variables and Si nutrition of rice were evident between Commerce silt loam and Gigger silt loam soil. While Si addition did not result in significant grain yield increase, there was a significant improvement observed on rice Si uptake. The survival test confirmed the presence of SSB in the different carriers, thus the observed improvement on straw Si content of rice can be associated with the use of SSB-inoculated carriers. Even so, this did not significantly increase rice biomass and grain yield. The lack of rice yield response to Si addition and SSB inoculation was partly explained by the high initial Si availability in both soil types. The semi-quantitative evaluation of silica bodies distribution on leaf surface of rice (treated with wollastonite and SSB using different carriers) via SEM-EDX further confirmed that the soil type had greater influence than Si addition and SSB inoculation on rice Si nutrition. More silica bodies were observed on the leaf surface of rice planted in Commerce silt loam than in Gigger silt loam. In the laboratory, the highest population of SSB was 5.0 x 10^6 cfu g^{-1} (log number of cells, 6.70) obtained in bagasse + soil carrier at 150 days after inoculation with a final population of 4.6 x 10^6 cfu g^{-1} at 180 days after inoculation (log

number of cells, 6.66). The fluorescent microscopy analysis showed that the green fluorescence protein tagged-SSB can colonize the root tissues of the two-week old rice seedlings indicating its ability to survive when used as a seed treatment, which is a very practical and efficient application method of potential bioinoculant to the field in the future. Future research will focus on (1) determining the optimum concentration of SSB to be inoculated in different carriers, and (2) evaluating the benefits of SSB application in varying field conditions.

Chapter 1. Introduction

Minerals are known as a nutrient reservoir in the soil (Uroz et al., 2007). With feldspar and mica being the main source of inorganic nutrients in soils, silicates are the most abundant minerals in the earth's crust (Chardon et al., 2006; Robert and Berthelin, 1986). Silicon (Si) is not recognized as an essential plant nutrient, but many crops have demonstrated its beneficial effects on plant growth and production, yield, and disease resistance (Sheng et al., 2008; Ma, 2004). Silicon, considering its abundance in the earth's crust, is mainly found in insoluble forms that are not readily available for plant uptake. It persists in an insoluble state until solubilized by weathering action of rocks or biological activity of plant roots and microorganisms (Naureen et al., 2015).

1.1. Silicon as an Element in Nature

Silicon commonly occurs in the form of silicates in nature, including ferromagnesian silicates (such as olivine, pyroxenes, and amphiboles), aluminosilicates (such as feldspar, mica, and clay), and silicon dioxide (e.g., amorphous silica, quartz). Silicon in silicate minerals is typically surrounded by four tetrahedral-style oxygen atoms. The amount of Si in soil ranges between 50 and 400 g Si per kg of soil (Balakhnina and Borkowska, 2013). Silicon compounds are primarily found in soil as SiO_2, around 50-70 percent of the soil mass, and in different types of aluminosilicate (Sommer et al., 2006). Liang et al. (2015) recorded that due to the presence of extremely active desilification and fertilization processes, Si content in latosols or latosolic red soils (highly weathered soils) in the tropical zone can be less than 1 percent. While Si is abundant in soil, most of its sources are not available because of the low solubility of Si compounds in soil for plant uptake.

Silicon is ranked as the second-most abundant element (after oxygen) in the earth's crust with nearly 29 percent mean content (Sommer et al., 2006). While Si is still not recognized as an essential nutrient for most plants, a wide variety of plants have been tested for the beneficial roles of this element in growth, production, yield and plant resistance to biotic stress (disease and pest) and abiotic stress (metal toxicity, nutrient imbalance, salt stress, extreme temperature, radiation and drought). Furthermore, Si has long been recognized as particularly important to rice (*Oryza sativa*) in the family Gramineae (Cai et al., 2008).

1.2. Role of Silicon in Plant Nutrition and Crop Protection

Silicon is an important beneficial nutrient element for the safe and competitive growth of all Asian cereals, including rice (Brunings et al., 2009). The role of Si in plant health and growth in Si-accumulating crops was investigated and was shown to be significantly effective (Jinab et al., 2008). Research shows that adequate Si uptake can boost the tolerance of agronomic crops, especially rice, to both abiotic and biotic stress (Ma and Takahashi, 2002). Although Si is not considered an essential element for higher plants, it has been shown to be beneficial for the growth and development of many plant species particularly tropical graminaceous plants which is a hyper-Si accumulator (Liang et al., 2007; Lee et al., 2019).

Silicon increases the resistance of plants to insect chewing, such as stem borer (*Scirpophaga incertulas*), by making plant tissue less digestible or by substantially harming the mandibles of feeding insects (Massey and Hartley, 2006). On the other hand, Si deficiency in plants makes them more vulnerable to insect feeding, fungal diseases, attacking germs, and abiotic stresses that adversely affect crop yield and quality. The vulnerability of rice to diseases such as rice blast (*Magnaporthe grisea*), leaf blight of rice (*Xanthomonas oryzae*), brown spot (*Cochliobolus miyabeanus*), stem rot (*Magnaporthe salvinii*) and grain discoloration has been shown to increase due to low Si uptake (Abed-Ashtiani et al., 2012). Further, their work recorded a decrease in blast intensity by 17-30% in rice planting regions of Colombia that was attributed to increasing Si concentration in plant tissues.

In several plant species, including rice, cucumbers (*Cucumis sativus*) and wheat (*Triticum aestivum*), as cited by Cai et al. (2008), Si-induced disease resistance has been observed. Silicon increases the resistance of rice to diseases like blast, sheath blight (*Rhizoctonia solani*), brown spot leaf scad, and stem rot. Silicon also increases plant tolerance to powdery mildew (*Blumeria graminis*) in wheat, barley (*Hordeum vulgare*), cucumber, and Arabidopsis. The Si-induced resistance mechanisms are, however, still not widely understood. Some research indicates that Si can accumulate in the leaves and thus interfere with the penetration of the pathogen through the mechanical barrier (Cai et al., 2008). Rapid development of defense-related enzymes such as peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) is thought to be an essential feature of plant response to invading pathogens. Phenylalanine ammonia-lyase is the main enzyme that determines the phenolic development rate via the phenylpropanoid pathway, while POD is involved in lignin biosynthesis (Cai et al., 2008). Cherif et al. (1994) reported that the introduction of Si into a hydroponics system improved POD, PPO, PAL and chitinase activities in *Pythium* spp. infected cucumber plants. Similar findings were also observed in wheat leaves contaminated by *Blumeria graminis* as cited by Cai et al. (2008).

Recently, the low available supply of Si was also considered as a primary contributor to cause “Localized Decline” in rice productivity in some locations in Louisiana (Breitenbeck et al., 2006). Silicon uptake has been reported to mitigate aluminum (Al) and iron (Fe) toxicity and a wide range of stresses in rice and other crops (Ma and Takahashi, 2002). Silicon fertilization, as cited by Babu et al. (2016), increased the number of tillers and grain yield of rice. Similarly, the application of calcium silicate in Histosols increased rice grain yields mainly because of the supply of available Si and not because of the supply of other nutrients. The effect of Si on disease reduction certainly has led to increased yields, but Si has also been shown to increase yield in the absence of disease.

Currie and Perry (2007) provided an alternative explanation for Si's protective function in plants stating its capability of activating a wide range of natural defenses. Enhanced activity of chitinases, PODs, PPOs and flavonoid phytoalexins was observed in Si-treated cucumbers, all of which can protect against fungal pathogens. Furthermore, metal toxicity, salinity, drought, and temperature stresses can be alleviated by Si application. Toxicities from excessive level of manganese (Mn), cadmium (Cd), Al, and zinc (Zn) are alleviated by Si through several mechanisms: 1) precipitation of metal as silicate, 2) reduction in lipid peroxidation, 3) increased activity of enzymatic (e.g., superoxide dismutase) and non-enzymatic (e.g., ascorbate) antioxidants, and 4) increased release of phenolics.

1.3. Silicon Accumulation in Rice

For more than half of the global population, rice is a model plant for genomic study of monocot species. Rice is also a typical siliciphilous plant, with the ability to metabolically absorb and store Si, although many upland crop plants tend to lack this ability. Numerous studies have shown that Si deposition in the plant tissues can increase the tolerance of rice to lodging, and biotic and abiotic stress. Silicon has long been known as a beneficial component of rice, but it has not been proven to be an essential element to all higher plants (Dai et al., 2005).

Rice is known as a Si accumulator and a crop requiring high Si (Ma and Takahashi, 2002). It accumulates Si at concentrations as high as 10% of shoot dry weight. The existence of specific Si transporters has been correlated with this accumulating capacity: in roots, Si is transported from the root epidermis to the root steels via Lsi1 and Lsi2 and then travels to the shoot with the transpirational water flow via the xylem sap (Ma et al., 2006). Lsi1 and LSi2 are examples of Si transporter. Lsi1, which belongs to the Aquaporin family of the NIP group, is responsible for the uptake of Si in both dicots and monocots from the soil into the root cells. Its expression patterns and cellular localization vary with plant species. The subsequent transport of Si out of the root cells towards the stele is governed by an active efflux transporter, Lsi2. Silicon is present in the xylem in the form of monosilicic acid and is discharged by Lsi6, a homolog of Lsi1 in rice (Yamaji et al., 2008). However, unlike Lsi1 and Lsi2, in addition to the root tips in rice, Lsi6 is also expressed in the leaf sheaths and leaf blades (Ma et al., 2011). Recent evidence indicates that three Si transporters (Lsi2, Lsi3 and Lsi6) located at the node are correlated with the distribution of Si to panicles and husk; knockouts of these transporters tend to decrease the distribution of Si to the panicles and increase Si levels in the flag leaf (Yamaji et al., 2015). Typically, silica is deposited in rice plants in the form of silica bodies, which are formed in epidermal cells, silica cells and bulliform cells (Kaufman et al., 1981).

Silicon depletion, which is a matter of concern, is correlated with rising rice yield per unit area (Savant et al., 1997). In several countries, Si fertilizer has been used to increase rice yield (Guntzer et al., 2012). Continuous mono-cropping with rice can significantly reduce plant-available Si in the soils in tropical and subtropical areas. In Vietnam, the practice of complete removal of straw during harvesting is as good as exporting Si from the field. Even so, the exogenous application of Si to rice in this region if often ignored. This indicates that Si may become a yield-limiting factor for the production of rice, so it may be essential to use exogenous Si fertilizer for an economic and sustainable rice production system (Ning et al., 2014).

1.4. Microorganisms in the Environment

In order to provide an alternative way to improve the cultivation and development of various crops and plants, plant-related microorganisms, especially bacteria and fungi, have been isolated and utilized for their plant growth-promoting properties. *Bacillus*, *Pseudomonas*, *Streptomyces, Burkholderia, Klebsiella, Azospirillium, Rhizobium, Trichoderma, Penicillium,* and *Aspergillus* are some of the most common microorganisms promoting plant growth. These microorganisms have been found to be associated with various crops such as rice, banana (*Musa acuminata*), tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), etc., and have been isolated from various sources, including soil, plant surface, and plant tissue (Masuchi and Komagata, 1988; Suddarma and Suprapta, 2011; Thangavelu and Mustaffa, 2012; Bhardwaj et al., 2014).

Gopalakrishnan et al. (2012) isolated numerous bacteria and fungi promoting plant growth from rice ecosystem. Four rhizobacteria (*Brevibacterium antiquum*, two strains of *Enterobacter ludwigii*, and *Pseudomonas monteilii*) were evaluated for their ability to promote rice growth. These strains were isolated from the rice rhizosphere of the rice intensification system (SRI). Inoculation of these strains significantly increased tiller numbers, stover, grain yields, total dry matter, root length, and volume and dry weight of rice. The microbial biomass carbon, N and dehydrogenase activity, total N, usable P, and organic carbon in the soil were also increased by these rhizobacteria. A total of 130 bacteria and 120 fungal isolates were isolated from the rhizosphere, rhizoplane, and pyllosphere of rice by Mwajita et al. (2013). Most of these isolated microorganisms can fix N, dissolve phosphate, and generate indole-3-acetic acid (IAA). The strains of *Pseudomonas* AF-7 and AF-1 increased the rice plant height and produced IAA, solubilized mineral phosphate, fixed N, and degraded propanil in propanil-contaminated rice fields (Procopio et al., 2012). *Bacillus methylotrophicus* sp. nov., strain CBMB205^T was isolated from the rhizosphere of rice and was found to use methanol (Madhaiyan et al., 2010).

Even in the acidic soils of the Brahmaputra Valley, Assamam, plant growth promoters were also isolated (Thakuria et al., 2004). Different bacterial strains were used such as *Azospirillum amazonense, Bacillus pantothenticus, Bacillus megaterium, Pseudomonas pieketti, Pseudomonas fluorescence,* which resulted in increased rice grain yield, nitrogenase activity, and IAA-like substances were also found to be produced. Plant growth-promoting rhizobacteria belonging to the genus *Enterobacter* and *Azospirillum* have also been used by Mehnaz et al. (2001) to promote rice growth.

1.5. Plant Growth-Promoting Bacteria and Silicate-Solubilizing Bacteria

Two new concepts, 'Biocontrol Plant Growth Promoting Bacteria (Biocontrol-PGPB)' and 'Plant Growth Promoting Bacteria (PGPB)', were proposed by Bashan and Holguin (1998), because many beneficial bacteria are not rhizosphere bacteria. Biocontrol PGPB is used to describe bacteria that either produce inhibitory substances or increase the natural resistance of the plant by suppressing a plant pathogen. The term PGPB refers to bacteria that promote growth by means other than the control of other microorganisms.

Plant Growth-Promoting Bacteria can influence plant growth directly or indirectly (Glick, 1995). By reducing or eliminating the deleterious effects of one or more phytopathogenic species, PGPB indirectly promotes plant growth (O'Sullivan and O'Gara, 1992; Cook, 1993; Glick, 1995). They can produce siderophores, antibiotics, hydrogen cyanide (HCN), and compete for nutrients as biocontrol agents (Kloepper, 1993). Direct effects include the production of substances that encourage plant growth, and increased nutrient release and plant uptake (Paterno, 2004).

One potential PGPB are those bacteria that can solubilize silica termed as silica-solubilizing bacteria (SSB). These bacteria solubilize insoluble forms of silicates hence increasing the supply of plant-available Si to plants. This increases Si uptake by plant, subsequently utilizing it (Si) to enhance plant defense mechanisms. Silicate solubilizing bacteria are found in soil, water, marine sediments and silicate minerals, but their number is lower than the overall bacteria, demonstrating their uniqueness (Vasanthi et al., 2018; Naureen et al., 2015).

1.5.1. Plant Growth- Promoting Compounds

1.5.1.1 Auxin

Plant hormones that both stimulate and inhibit the growth of plants are called auxins. The most frequently found natural auxin is IAA. Auxins have been found responsible for phototropism, geotropism, and apical superiority.

Auxin is the most important plant growth regulator. It plays an important role in flower initiation and root formation. It is also responsible for raising the fruit size of many young fruits and is also important for fruit ripening. The first practical application of growth substance is observed in root formation due to application of auxins. Auxin is the first known plant growth regulator which used in the plant industry. Another auxin feature that is important in plant propagation is the rooting of cuttings (Weaver, 1917).

Auxins are present in plant roots as well as in shoots. The studies of Thimann (1969) offered proof that roots are much more sensitive than shoots to auxin. Development in roots could be inhibited by the same amount of auxin that encourages cell elongation in shoots.

The capacity of auxin to both stimulate and inhibit the growth of plants explains why shoots grow upward and roots grow downward. When a stem is placed in a horizontal position, the lower side of the plant accumulates auxin at higher concentrations. The auxin allows the cells to elongate more rapidly on the underside of the shoot than the cells on the upper side, so the shoot grows upward. Although several hypotheses exist, the mechanism of auxin in action is not completely understood. The presence of auxin has been associated with ethylene production in plants. Since some of the responses associated with auxins can be duplicated by exposing the plants to ethylene, the idea is that the growth responses attributed to auxins actually result from ethylene produced by the auxin. Another hypothesis is that auxin enhances the cell walls' plasticity; it enables the cells to stretch to greater sizes. Auxin increases the supply of energy in the tissue where it is present and induces plant growth with this increased metabolic activity (Ingles, 1994).

1.5.1.2. ACC-deaminase Activity

Ethylene is a major stress hormone, since a number of stresses trigger its synthesis. The immediate precursor of ethylene in larger plants is 1-aminocyclopropane-1-carboxylate (ACC). Due to their ACC-deaminase activity, some rhizobacteria can hydrolyze ACC into ammonia and alfa-ketobutyrate (Morgan and Drew, 1997).

The gaseous plant hormone ethylene is involved in the life cycle of plants via regulation of many developmental processes (Abeles et al., 1992; Reid, 1995). Ethylene is often considered a stress hormone, as a number of stress signals such as mechanical wounds, chemicals and metals, drought, high temperatures, and pathogen infection cause its synthesis (Morgan and Drew, 1997).

1-Aminocyclopropane-1-carboxylate in higher plants is an immediate ethylene precursor. Ethylene development in plants is heavily dependent on ACC endogenous levels (Lurssen et al., 1979; McKeon et al., 1982). Therefore, ACC accumulates simultaneously with a rapid burst in ethylene production in the early stages of plant stress response (Morgan and Drew, 1997).

Some microorganisms have been found to produce the enzyme ACC deaminase which, instead of converting it into ethylene, hydrolyses ACC into ammonia and alpha-ketobutyrate (Glick et al., 1994a, b; 1998; Mayak et al.,1999; Shaharoona et al., 2006a). The uptake of ACC and cleavage by rhizobacteria containing ACC-deaminase decreases the amount of ACC, as well as ethylene, in the roots, thereby acting as a sink for ACC. Decreased ACC levels result in lower levels of endogenous ethylene, reducing the possibly inhibitory effects of higher ethylene concentrations caused by stress (Glick et al., 1998).

Rhizobacteria containing ACC deaminase can increase root growth by decreasing endogenous levels of ACC (Glick, 2005). Bacteria lacking ACC deaminase, however, have also been shown to increase plant growth, and known mechanisms cannot explain such observations. Bacterial cells are assumed to possess certain surface components under such conditions, or to secrete compounds that serve as 'elicitors' of plant growth. Plant roots must be able, in ways similar to the identification of elicitors from plant pathogens, to perceive and identify such elicitors. In fact, by being perceived by similar receptors, plant pathogens might interfere with the action of plant growth-promoting rhizobacteria (PGPR). Recently, several studies have suggested that inoculation with ACC-deaminase-containing rhizobacteria increased the growth of inoculated plants under gnotobiotic conditions, mainly through the control of ethylene synthesis in the inoculated roots. In addition, plants treated with ACC-deaminase-containing PGPR are significantly more resistant to the deleterious effects of stress ethylene synthesized as a result of stressful conditions such as flooding (Burd et al., 1998), drought, and elevated level of salt.

1.5.1.3. Phosphate solubilization

Many soil microorganisms are potential solubilizers of bound phosphates. A variety of organic acids such as formic, acetic, propionic, lactic, glycolic, fumaric, and succinic acid are known to reduce the pH of the substrate by secreting phosphate-dissolving bacteria (Subba Rao, 1999). Many reports on the use of P dissolving bacteria showed increase of plant growth, but some results were not associated to P solubilization. It indicates that other mechanisms were responsible for the positive growth response of plants (Pietr et al., 1991; Berthelin et al., 1991; De Freitas et al., 1997).

The organic acids release by phosphate solubilizing bacteria can dissolve insoluble mineral phosphates (Berthelin et al., 1991). Rhizospheric microbes release usable phosphate from the rock phosphate which helps on promoting plant growth (Pietr, 1991). A research performed by Louw and Webley (1958 and 1959) showed that available form of P from Gafsa rock phosphate and calcium phosphate were made accessible by phosphate-dissolving isolates. None of the isolates examined, however, released phosphate from variscite and strengite or taranakite, respectively.

The ability of phosphate-dissolving bacteria to dissolve bound phosphate can be achieved by using dicalcium phosphate or tricalcium phosphate-containing agar media (Louw and Webley, 1959; Goldstein, 1986; Subba Rao, 1999). On the surface of the medium, the bacteria are streaked, and the presence of a clear zone around the growth suggests positive P-solubilization. Soluble phosphate estimation (expressed as P_2O_5) can be done with the colometric method defined by Williams and Stewart. Paper chromatography and the use of solvents and sprays for the identification of acids formed by P-dissolving bacteria are other techniques (Louw and Webley, 1959).

1.5.1.4. Nitrogen Fixation

Intensive analysis of this mechanism has been motivated by the significance of biological N_2 fixation. The growth of all species is based on nitrogen (N) availability. The roots and rhizospheres of several plant species can be colonized by a variety of free-living diazotrophs and make small amounts of fixed N accessible by plants (Zuberer, 1998). It includes aerobic bacteria, facultative aerobic bacteria, and anaerobic bacteria. The N-fixing capacity is only present in some bacteria. Some live with plants in a symbiotic relationship, and some live free in the soil. Nitrogen-fixing cyanobacteria are essential to maintaining the fertility of semi-aquatic environments like rice paddies.

1.6. Molecular Characterization of Bacteria

The most powerful approaches to taxonomy are through the studies of nucleic acids. The significance of phylogenetic studies based on 16S rDNA sequences is increasing in the systematics of bacteria. Sequences of 16S ribosomal DNA have provided bacteriologists with a phylogenetic tree that allows the investigation of evolution of bacteria and also provides the basis for identification.

The 16S rDNA research starts by isolating DNA and using polymerase chain reaction (PCR) to amplify the gene coding for 16S rRNA. The DNA fragments that are purified are sequenced directly. In order to determine the order in which the bases are arranged within the length of the sample, the sequencing reactions are carried out using a DNA sequencer, and a computer is then used to analyze the sequence for identification using phylogenetic analysis procedures (Boudemagh, 2005).

1.6.1. Polymerase Chain Reaction

Nucleic acids are analyzed by the majority of molecular methods currently in use for population research. Some of these techniques analyze nucleic acids directly, while PCR amplification has been used for easier detection to increase copies of a target gene.

Polymerase chain reaction is an in-vitro DNA sequence amplification process. It helps researchers to generate millions of copies in approximately two hours of a particular DNA sequence. This process increases the amount of a DNA sequence from hundreds of millions to billions of times, beginning with DNA of any origin-bacterial, viral, plant, or animal. The need to use bacteria to amplify DNA is bypassed by this automated procedure. Polymerase chain reaction has revolutionized methodologies in molecular biology (Pepper, 1999). It is an enzymatic mechanism carried out in discrete amplification cycles. Both of which can double in the sample the amount of target DNA. Thus, n cycles can produce 2n times as much target as was present to begin with (Arnheim and Levenson, 1990). Researchers also choose PCR primers to target particular populations known to be major soil inhabitants, such as actinomycetes, due to the enormous complexity of soil communities (Heuer et al., 1997; Nakatsu et al., 2000).

1.7. Economic Importance of Rice

Rice is one of the most significant cereal crops in the world, with about 154 million hectares harvested per year. As cited by Cuong et al. (2017), it is the main source of calorie intake and the staple food of more than three billion people in the world. It nourishes more than half the population of the planet (Zhao et al., 2010; Zhang et al., 2011). Owing to an increase in the global population, the demand for rice is steadily growing. However, certain constraints such as water shortage, infestation with pests, insufficient use of fertilizers and growth of conventional low-yielding varieties limit yield increases (Datta et al., 2017). Asian countries are the main consumers of rice, considering rice as the staple food for more than 1.3 billion people.

Rice cultivation has been going on for more than 10,000 years (Zhao et al., 2010). The plant is currently widely cultivated worldwide, especially in Asia (Jena and Mackill, 2008). Around 90 percent of the 576 million tons of rice grown worldwide in 2002 were provided by Asian countries. China and India normally produce about 50 percent of the world's rice together. In more than 50 other nations, it is a major agricultural crop. In the countries where it is made, about 96 percent of the rice grown worldwide is consumed, with some exceptions.

Various attempts have been made to cultivate and grow rice effectively. The production of rice is limited by various abiotic and biotic variables. Therefore, various methods and practices are used to continue rice cultivation in a wide range of conditions, particularly in stress-causing and harmful environments. Researchers and farmers currently use chemicals such as fertilizers, pesticides, fungicides and bactericides, and breed varieties that are abiotic stress-tolerant and biotic stress-resistant and other methods to safeguard and maintain the production of rice.

In terms of farm acreage and economic value, rice is one of Louisiana's most valuable crops. Its production has spread to the northeastern region of the state from the traditional rice growing areas of southwestern Louisiana. Under both aerobic and anaerobic conditions, it can be cultivated. The United States exported approximately 37 percent of the 8.7 million tons it produced in 2000, according to the FAO, and Pakistan exported approximately 28 percent of its 7.2 million tons. Thailand exported considerably more rice than any other country in the same year, 6.6 million tons, or about 26% of its total, while India exported 1.5 million tons, or about

1.1% of its total production. Major rice-importing countries include Nigeria, Philippines, Iran, Saudi Arabia, Brazil, Senegal, Japan, and Indonesia (Hines, 2009).

1.8. Rationale for Research

Environmental issues, increasing chemical fertilizer prices and the need to boost crop production have prompted researchers to conduct studies to develop products that can improve the productivity of rice crops. In recent years, the use of plant growth regulators to adjust the variables regulating all stages of crop growth from seed germination through vegetative growth, maturity, senescence, aging and post-harvest preservation has become more prevalent (Grzesik and Rudnicki, 2002). There have been several developments in the practical use of plant growth substances alongside fundamental research at the biochemical, physiological, and molecular levels. In agriculture, the use of these compounds has a great deal of potential to control many, if not all, physiological processes in plants.

The soil has an important role in the survival and growth of plants as the nutrients needed and used for survival are available in the soil. In addition, the soil is populated by multitudes of organisms that are very influential in the survival of the plant, such as bacteria, actinomycetes, fungi, algae, protozoa, molds, viruses, and various small animals and insects (Siddiqui et al., 2009; Siddiqui et al., 2011).

Different microorganisms play an important role in maintaining the quality of both the plant and the soil (Doni et al., 2014). Soils with a high biodiversity of species are shown to be more stress tolerant compared to soils with low biodiversity that have compromised ecosystem function (Tilman et al., 1997; Griffiths et al., 2000; Hamayun et al., 2010). The rhizosphere, from the Greek word ‘rhizo’ or ‘rhiza’, means root and sphere, is the zone or area where the soil is under the influence of the plant’s roots. It is the region where the root exudates generate simple and complex sugars, growth regulators, amino acids, phenolic acids, flavonoids, fatty acids, enzymes, steroids, alkaloids, vitamins, etc., where intense microbial activity occurs, enabling the establishment of multiple microbial communities (Hiltner, 1904; Sidduqui et al., 2009; Parvin Joshi et al., 2011; Gopalakrishnan et al., 2012; Mwajita et al., 2013).

Plant growth-promoting rhizobacteria are the bacteria that actively colonize the rhizosphere and exert beneficial effects on plant growth (Schroth and Han Cock, 1982; Kloepper and Schroth, 1978). Known as PGP, beneficial bacteria affect plant growth and survival through direct and indirect mechanisms (Gopalakrishnan et al., 2012). Direct stimulation may include fixation of N, development of siderophores, solubilization of phosphate, production of IAA, and production of various enzymes and phytohormones. Indirect mechanisms involve the induction by various mechanisms, such as antibiotic production, siderophore production, parasitism, and competition of nutrients. Via increased biomass, germination rates, leaf area, chlorophyll content, N content, protein content, hydraulic activity, roots and shot length, yield and tolerance to various abiotic stresses such as salinity and flooding, these can affect the growth of the plant (Thakuria et al., 2004; Siddiqui et al., 2009; Siddiqui et al., 2011; Jha et al., 2013). The isolation, detection, classification and use of beneficial bacteria would serve as a great alternative to traditional methods in order to provide a safer and more sustainable way of growing and producing rice and other plants, because these microorganisms are already present in the environment.

Silicate-solubilizing bacteria is a potential PGPB which plays an effective function in solubilizing insoluble types of silicates thereby increasing soil fertility and improving plant defense mechanisms. The solubilized silica in the form of orthosilicic acid (H_4SiO_4) is absorbed along with water. Silicon is accumulated in the form of silica gel and is deposited in epidermal cells, sclerenchyma, vascular bundles, and in florescence brackets in cereals. The accumulated Si not only improves growth and yield of these plants but is also involved in induction of systemic resistance (ISR) against pests and diseases (Naureen et al., 2015). The objectives of this research were to: 1) isolate and profile SSB in Louisiana soils, 2) investigate the effect of SSB on the absorption of Si and the productivity of rice using various carriers, and 3) develop a feasible approach of incorporating SSB in the rice production system.

Chapter 2: Isolation and Profiling of Silicate-Solubilizing Bacteria in Louisiana Soils

2.1. Introduction

Minerals are considered as a reservoir of nutrients in the soil (Uroz et al., 2007). Silicates are the most common minerals in the earth's crust with feldspar and mica being the main source of inorganic nutrients in soils (Chardon et al., 2006; Robert and Berthelin, 1986). Silicon (Si) is not recognized as an essential nutrient for plants, however its beneficial effects on plant growth and development, yield, and disease resistance have been documented in many crops (Sheng et al., 2008; Ma, 2004).

Silicon increases the growth and yield of several plants and reduces the occurrence of many plant diseases in various pathosystems, such as fungal and bacterial diseases. It strengthens the cell walls and the outer membrane of leaf epidermal cells, and activates the deployment of natural plant natural defenses, thereby preventing the penetration of pathogenic fungi (Fauteux et al., 2005). Naureen et al. (2015) reported that Si improves plant growth, increases rigidity of leaves thus maximizing surface area for photosynthesis and mitigates the effects of abiotic stresses such as drought, salt and metal toxicity in several plants including wheat (*Triticum aestivum)*, rice (*Oryza sativa*), sugarcane (*Saccharum officinarum*), cucumber (*Cucumis sativus*), tomato (*Solanum lycopersicum* L.), citrus (*Citrus limon*) and barley (*Hordeum vulgare*).

Silicon is mostly present in insoluble forms that are not readily available for plant uptake, despite of its abundance on the earth's crust. It remains in insoluble form unless solubilized by weathering action of rocks or biological activity of plant roots and microorganisms (Naureen et al., 2015). Studies have shown that degradation of silicate minerals by bacteria release potassium (K) and Si (Sheng and He, 2006; Hutchens et al., 2003; Welch et al., 1999; Barker et al., 1998).

Microorganisms play a vital role on the dissolution of soil minerals by different mechanisms especially in ion cycling and soil fertility (Uroz et al., 2007; Calvaruso et al., 2006; Ehrlich, 1996). Soil beneficial microorganisms such as nitrogen (N) -fixers and phosphate-solubilizing bacteria (PSB) are effective plant growth-promoters. These groups of bacteria control the chemical and biological properties of soil through absorption of soluble monosilicic acid. Another group of bacteria, silicate-solubilizing bacteria (SSB), is involved in the conversion of silicates into soluble silica (Rangaraj et al., 2013). Naturally occurring Si can be depolymerized and solubilized by bacteria. Several mechanisms of silicate disintegration by bacteria have been proposed, which include solubilization by ligands (divalent cations), acids (organic and inorganic), alkali (nucleophilic attack) and extracellular polysaccharides. Nevertheless, acidolysis is acknowledged as the most commonly occurring mechanism by which silicate minerals are weathered (Jongmans et al., 1997).

Silicate-solubilizing bacteria play an important role in solubilizing insoluble forms of silicates hence increasing soil fertility and enhancing plant defense mechanisms. Silicate-solubilizing bacteria are distributed in soil, water, aquatic sediments and in silicate minerals however their population is smaller than the total bacteria indicating their uniqueness (Vasanthi et al., 2018; Naureen et al., 2015). Despite that bacteria are known to facilitate mineral weathering processes, however, the characteristics of mineral-solubilizing bacteria from weathered feldspar surfaces and the mechanisms by which bacteria exert their influence are still uncertain (Wightman and Fein, 2004; Lee and Fein, 2000). A comprehensive evaluation of biological components of the soil and the possible utilization mechanisms of different minerals, such as silica, is lacking (Rangaraj et al., 2013). Reports on the population and diversity of SSB and the changes in soil silica contents are scanty. Profiling of SSB is one way of determining its diversity or population in the soil. Quantifying silica content in both soil and plants are also essential in assessing the effectiveness of SSB in plant growth.

The general objective of this study was to isolate and profile SSB in Louisiana soils. Specifically, the objectives of the study were the following:

a. Identify the species of SSB in soils typically grown to field crops (e.g. rice, corn (*Zea mays*), soybean (*Glycine max*), and wheat) in Louisiana
b. Identify and characterize SSB using morphological and molecular analysis
c. Profile other growth-promoting compounds released by SSB

2.2. Materials and Methods

Laboratory activities included isolation, screening, and identification of SSB using morphological, biochemical, and molecular techniques. It also included screening of SSB isolates for their growth-promoting activities, such as indole-3-acetic acid (IAA) production, phosphate solubilization, N fixation, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity (Appendix Figure 2.1).

2.2.1. Time and Place of Study

The laboratory experiment was conducted from October 2018 to October 2019 at the laboratory facilities of the School of Plant, Environmental, and Soil Sciences, and Department of Plant Pathology and Crop Physiology at Louisiana State University campus in Baton Rouge.

2.2.2. Isolation of Silica-Solubilizing Bacteria

2.2.2.1. Collection of Soil Samples for Isolation of Silica-Solubilizing Bacteria

The soils were collected from fields grown to soybean, wheat, corn, sugarcane, and rice at various locations in Louisiana (Figure 2.1). The details of the sources were given in Table 2.1.

About a kg of soil sample was collected from the top 0-15 cm of at least 20 sampling points using a JMC 36” Soil Sampler. Each sample was placed in clean paper bags. The collected samples were processed immediately for isolation of bacteria. Ten grams of soil sample were weighed and were used for isolation (Shinde, 2014).

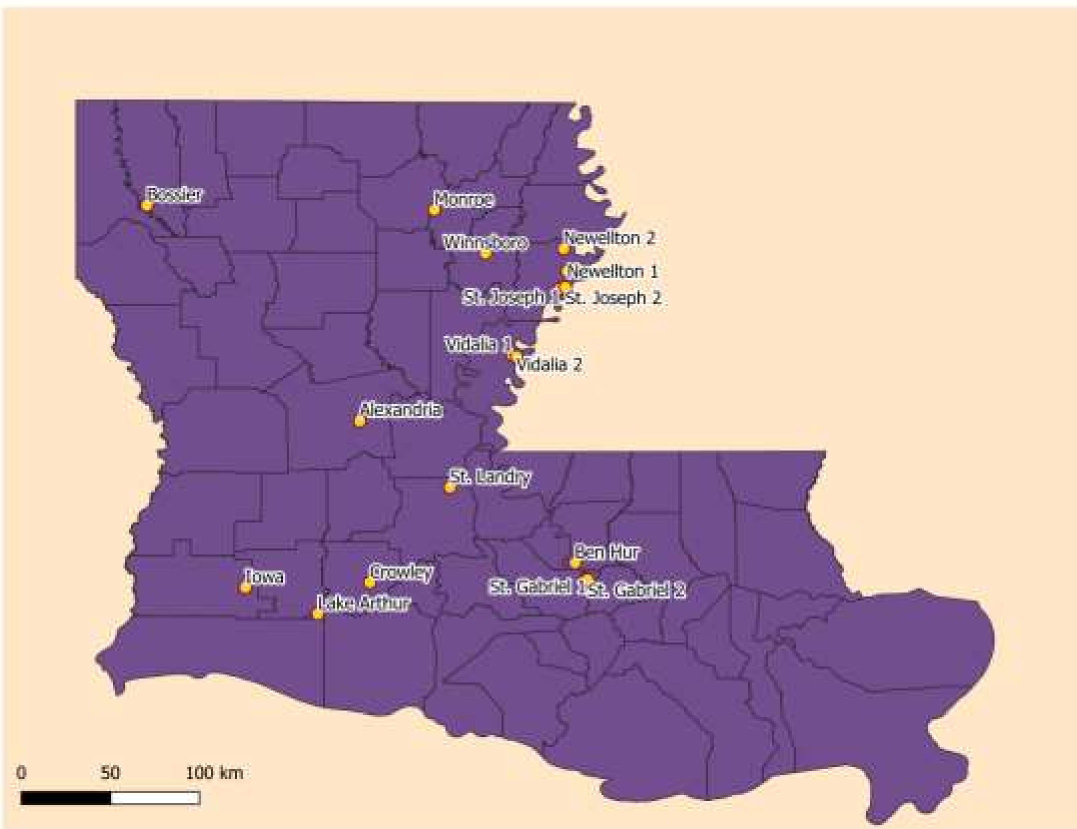


Figure 2.1. Map of Louisiana showing the seventeen soil sampling sites.

Table 2.1. Type and general properties of soils that were collected from different locations in Louisiana for isolation and profiling of SSB.

Crop	Location	Coordinates	Soil Type	Soil Classification	General Soil Chemical Properties (e.g. pH, OM[‡], soil Si[†])
Soybean-Corn	Bossier	32.4179, -93.637718	Latanier clay	Clayey over loamy, over smectitic mixed, superactive, thermic Oxyaquic Hapluderts	low pH
Rice	Crowley	30.2462, -92.3515	Crowley silt loam	Fine, smectitic, thermic Typic Albaqualfs	high pH, medium OM, medium Si
Rice	Lake Arthur	30.0657, -92.6520	Kaplan silt loam	Fine, smectitic, thermic Aeric Chromic Vertic Epiaqualfs	low pH
Sugarcane	St. Gabriel 1	30.26833, -91.10556	Mixed: Sharkey clay and Gramercy silty clay loam	Very-fine, smectitic, thermic Chromic Epiaquerts Fine, smectitic, hyperthermic Chromic Epiaquerts	low pH, medium OM, medium Si
Sugarcane	St. Gabriel 2	30.2625, -91.09722	Commerce silt loam	Fine-silty, mixed, superactive, nonacid, thermic Fluvaquentic Endoaquepts	neutral pH, medium OM, medium Si
Soybean/corn	Ben Hur	30.36, -91.17	Cancienne silt loam	Fine-silty, mixed, superactive, nonacid, hyperthermic Fluvaquentic Epiaquepts	low pH
Rice-soybean	Iowa	30.21813, -93.06925	Crowley-Vidrine complex;	Fine, smectitic, thermic Typic Albaqualfs	low pH
Soybean-Corn	Winnsboro	32.1418, -91.6862	Gigger-Gilbert complex, gently undulating	Fine-silty, mixed, active, thermic Typic Fragiudalfs	low pH, low OM, low Si

(Table 2.1 Continued).

Rice	Monroe	32.3913, -91.9817	Hebert silty clay loam	Fine-silty, mixed, active, thermic Aeric Epiaqualfs	low pH
Cotton-Corn	Newellton 1	32.0375932, -91.2151406	Bruin silt loam	Coarse-silty, mixed, superactive, thermic Oxyaquic Eutrudepts	low pH
Soybean/wheat /corn	Alexandria	31.17694, -92.40972	Coushatta silt loam	Fine-silty, mixed, superactive, thermic Fluventic Eutrudepts	high pH, low OM, low Si
Soybean-Corn	Newellton 2	32.1676, -91.2359	Sharkey clay	Very-fine, smectitic, thermic Chromic Epiaquerts	neutral pH
Rice	St. Landry	30.79497, -91.89164	Tensas-Sharkey complex, gently undulating	Fine, smectitic, thermic Chromic Vertic Epiaqualfs	neutral pH
Rice	Vidalia 1	31.5541, -91.5323	Sharkey clay	Very-fine, smectitic, thermic Chromic Epiaquerts	high pH, medium OM, high Si
Corn-Soybean	Vidalia 2	31.5461, -91.5077	Commerce silt loam	Fine-silty, mixed, superactive, nonacid, thermic Fluvaquentic Endoaquepts	low pH
Rice-Rice	St. Joseph 1	31.9359003, -91.2573157	Sharkey clay	Very-fine, smectitic, thermic Chromic Epiaquerts	neutral pH
Rice	St. Joseph 2	31.94522, -91.22589	Sharkey-Tunica-Newellton complex, gently undulating	Very-fine, smectitic, thermic Chromic Epiaquerts	neutral pH

‡ - organic matter content

† - soil Si based on 0.5 M acetic acid procedure; information is from previous studies (Paye et al., 2018; Babu, 2015).

2.2.2.2. Culture Media

The following media were used: Luria broth (LB) agar, tryptic soy agar (TSA), silica broth and agar medium. These were in dehydrated form that were prepared only when needed. All media were sterilized in an autoclave for 20 min at 121°C before use. The bacterial isolates from the soil collected in different sites were subjected to a silicate-solubilizing test. Briefly, each bacterial isolate was incubated on silicate medium (10 g L^{-1} glucose, 2.5 g L^{-1} magnesium trisilicate [$Mg_2O_8Si_3$], and 20 g L^{-1} agar) (Kang et al., 2017).

2.2.2.3. Isolation and Purification

Ten grams of fresh soil sample were transferred to a 250 ml Erlenmeyer flask containing 100 mL of sterile distilled water. The samples were placed on a rotary shaker (130 rpm) for 3 days at 37˚C. After 3 days of incubation, serial dilutions of each 100 mL enriched sterile distilled water, ranging from 10^{-1} to 10^{-6} was prepared in 9 ml sterile distilled water. One mL aliquot of the appropriate dilution was spread on plates containing silica agar (Kang et al., 2017). Plates were incubated at 37˚C ± 1˚C for 24 - 48 h and colonies were examined. Morphologically distinct colonies were counted, selected, purified, and maintained in silica agar (Naureen et al., 2015; Shinde, 2014).

2.2.2.4. Measurement of Silicate Solubilizing Zone

Bacterial isolates were spot-inoculated on silicate medium. Plates were incubated at 37˚C ± 1˚C for 24 - 48 h and clearing around colonies was measured. Solubilization Index (SI) expressed as halo diameter (mm)/colony diameter (mm) was calculated (Akintokun et al., 2007). Silicate-solubilizing bacteria isolates were grouped based on their SI as demonstrating low ($SI < 2.00$), intermediate ($2.00 < SI < 4.00$) and high ($SI > 4.0$) solubilization capacities (Santi and Goenadi, 2017).

2.2.3. *In vitro* Screening of SSB Isolates for Growth-Promoting Activities (GPA)

2.2.3.1. Indole-3-acetic acid production

Isolates were grown in tryptic soy broth (TSB) supplemented with tryptophan (Kang *et al*., 2017). All media such as LB, TSA, silica broth and agar were sterilized for 20 min at 121°C and 15 psi before use. After 7 days of incubation, the cultures were centrifuged at 13,000 rpm for 10 minutes. One milliliter of the supernatant was mixed with 2 ml of Salkowski reagent and the appearance of a pink color indicated IAA production. The absorbance was measured in a Coleman Mod. 14 spectrophotometer at 530 nm and the quantity of IAA produced was estimated against the IAA standard (Gordon and Weber, 1951).

2.2.3.2. Phosphate solubilization

The isolates were grown in the solid media containing precipitated tricalcium phosphate. It was sterilized for 20 min at 121°C and 15 psi before use. The medium that was used is modifications of Pikovskaya's medium (Subba Rao, 1999). The bacterial isolates were spot-inoculated onto the surface of the agar. The presence of a clearing zone around the bacterial growth as indication of P-solubilization was noted after 7 days of incubation.

Phosphate-solubilizing capacity of isolates was semi-quantitatively determined in terms of its phosphorus solubilization index (PSI) using Equation 1 by Islam and Hossain (2012). Using desk ruler, total diameter of halo zone was measured as the length of the halo zone from one edge to the other. Same procedure was followed for measuring colony diameter.

Eq 1. $$PSI = \frac{\text{total diameter of the halo zone (mm)}}{\text{colony diameter (mm)}}$$

2.2.3.3. 1-Aminocyclopropane-1-carboxylic acid-deaminase activity

The isolates were grown using the N-free Dworkin and Foster's (1958) salts minimal agar medium. The medium was supplemented with 3 mM ACC (Sigma) per liter as a sole N source. It was sterilized for 20 min at 121°C and 15 psi before use. Five-day-old isolates grown on TSA were streaked on Dworkin and Foster (DF) agar medium plates amended with ACC. The plates were incubated at 28+/-2°C in the dark for 7 days. Growth and sporulation of the isolates on DF agar medium amended with ACC (DF-ACC agar) were taken as indicators of the efficiency of selected isolates to utilize ACC and to produce ACC deaminase.

2.2.3.4. Nitrogen fixation

Jensen's N-Free medium was used for the screening of free living N_2-fixing bacteria. Hussain and Srinivas (2013) used Jensen's medium for the isolation of N-fixing bacteria. Cruz et al. (2016) also used this selective medium for the isolation of endophytic N-fixers in nipa (*Nypa fruiticans*). Growth of the bacteria in this selective medium was taken as an indicator that the bacteria are potential N-fixers. This medium was only prepared when needed. The media was sterilized for 20 min under 121° C and 15 psi before use.

2.2.4. Identification of Silicate-Solubilizing Bacterial Isolates

2.2.4.1. Morphological Identification

Gram staining was conducted to determine if the isolates are gram positive or gram negative bacteria. Gram-positive cells have a thick peptidoglycan layer and stain blue to purple. On the other hand, Gram-negative cells have a thin peptidoglycan layer and stain red to pink (Smith and Hussey, 2012). Peptidoglycan serves a structural role in the bacterial cell wall, giving structural strength, as well as counteracting the osmotic pressure of the cytoplasm.

2.2.4.2. Molecular Identification using 16S Ribosomal deoxyribonucleic acid (DNA) Analysis

2.2.4.2.1. Isolation of genomic DNA

Isolates were grown in TSB medium for 1 day. Then, the DNA was extracted from the isolates using Qiagen DNA extraction kit.

2.2.4.2.2. Polymerase chain reaction (PCR) amplification of 16S ribosomal DNA

Polymerase chain reaction run was performed using a BIO-RAD MyCycler^{TM} thermal cycler. Near full-length 16S ribosomal DNA (approximately 1.5 Kb in length) was amplified through PCR using bacteria-specific forward primer F2D 308 and universal reverse primer RD2 312 (Table 2.2). The 25 µL reaction mixture contains: 5 µL 5X PCR buffer; 0.5 µL 10mM dNTPs; 0.2 µL Q5 high fidelity DNA Polymerase; 5 µL Q5 Enhancer; 1.0 µL forward primer F2D 308; 1.0 µL reverse primer RD2 312; 1.0 µL DNA template; and 11.3 µL double distilled H_2O. The thermocycling program consists of one cycle of initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, and extension at 72°C for 1 minute and 45 seconds, and one cycle of final extension at 72°C for 30 minutes with a holding temperature of 10°C for temporary storage of the reaction.

Eight microliter aliquots of all PCR products were electrophoresed in 1.0 % agarose gel in 10X Tris-Borate-EDTA (TBE) buffer at 100 V for approximately 30 minutes using Mupid® submarine electrophoresis system (Advance Corporation, Chuoku, Tokyo, Japan) and were viewed under UV using UVP Doc It® gel documentation system (UVP, LLC, Upland, California, USA) after staining with ethidium bromide.

2.2.4.2.3. Sequence analysis

The PCR products were sent to Macrogen, Inc. for sequencing. Sequences were edited using BioEdit v 7.0.5 then the DNA sequences were checked for homology to bacteria-specific genes using the BLAST program of the NCBI-BLAST website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Software MEGA 10.0.5 was then used to align the corresponding sequences of representatives within the related genera *Pseudomonas, Bacillus, Aeromonas* and *Enterobacter*, which were retrieved from the GenBank databases.

Table 2.2. Oligonucleotide primers used in the amplification of 16S ribosomal DNA.

PRIMER	TARGET GROUP	SEQUENCE (5' -3')
308F	Bacteria	CCG AAT TCG TCG ACA ACA
	Forward	GAG TTT GAT CAT GGC TCA G
312R	Universal	CCC GGG ATC CAA GCT TAC
	Reverse	GGC TAC CTT GTT ACG ACT T

The program used for alignment of sequences was MAFFT (Multiple Alignment using Fast Fourier Transform) v 7.0. In bioinformatics, MAFFT is a program used to create multiple sequence alignments of amino acid or nucleotide sequences. Gblocks v 0.91b was then used to treat and select conserved blocks from multiple alignments for their use in phylogenetic analysis (Talavera and Castresana, 2007). Phylogenetic trees were inferred with PhyML 3.0: new algorithms, methods and utilities for the maximum likelihood tree (Guindon et al., 2010).

2.3. Results and Discussion

2.3.1. Isolation of Silicate-Solubilizing Bacteria

One hundred thirty bacteria were isolated from the soils collected at different locations in Louisiana (Table 2.3). Most of these soils are currently cultivated for soybean, wheat, corn, sugarcane, cotton, and rice production. Out of the 130 bacteria only twenty were able to solubilize silicate as indicated by a clearing zone around bacterial colony. (Figure 2.2).

The highest percentage of SSB was observed in soil samples collected from St. Gabriel wherein 67% of the bacterial isolates were silicate-solubilizers. This location has been under routine cultivation of sugarcane. On the other hand, the percentage of SSB in Iowa and Bossier was 43% and 40%, respectively. The field in Bossier had been under routine cultivation for soybean-corn production while the field in Iowa has been under rice-soybean production. In Crowley, 20% of the bacteria population isolated was able to solubilize silicate. The same percentage of silicate-solubilizers (17%) was obtained from Newellton 2, Vidalia 1, and Vidalia 2. A few silicate solubilizers ranging from 7-14% were seen in soil samples from Winnsboro, Newellton 1, Alexandria, St. Joseph 2 and St. Landry. No silicate-solubilizers were found in Ben Hur, Lake Arthur, Monroe and St. Joseph 1.

Table 2.3. List of bacteria isolated from soils collected in different places in Louisiana.

Location	Bacterial Isolates		Silicate Solubilization
	No.	Code	
Bossier	1	Bossier 1	-
	2	Bossier 2	-
	3	Bossier 3	-
	4	Bossier 4	-
	5	Bossier 5	-
	6	Bossier 6	-
	7	Bossier 7	+
	8	Bossier 8	+
	9	Bossier 9	+
	10	Bossier 10	+
Crowley	11	Crowley 11	-
	12	Crowley 12	-
	13	Crowley 13	-
	14	Crowley 14	-
	15	Crowley 15	-
	16	Crowley 16	-
	17	Crowley 17	-
	18	Crowley 18	-
	19	Crowley 19	+
	20	Crowley 20	+
Lake Arthur	21	Lake Arthur 21	-
	22	Lake Arthur 22	-
St. Gabriel 1	23	St. Gabriel 23	+
	24	St. Gabriel 24	+
	25	St. Gabriel 25	+
St. Gabriel 2	26	St. Gabriel 26	+
	27	St. Gabriel 27	-
	28	St. Gabriel 28	-
Ben Hur	29	Ben Hur 29	-
	30	Ben Hur 30	-
	31	Ben Hur 31	-
	32	Ben Hur 32	-
	33	Ben Hur 33	-
	34	Ben Hur 34	-
	35	Ben Hur 35	-
	36	Ben Hur 36	-
	37	Ben Hur 37	-
	38	Ben Hur 38	-

(Table 2.3 Continued).

	39	Ben Hur 39	-
	40	Ben Hur 40	-
	41	Ben Hur 41	-
Iowa	42	Iowa 42	-
	43	Iowa 43	-
	44	Iowa 44	+
	45	Iowa 45	-
	46	Iowa 46	+
	47	Iowa 47	+
	48	Iowa 48	-
Winnsboro	49	Winnsboro 49	-
	50	Winnsboro 50	-
	51	Winnsboro 51	-
	52	Winnsboro 52	-
	53	Winnsboro 53	-
	54	Winnsboro 54	+
	55	Winnsboro 55	-
Monroe	56	Monroe 56	-
	57	Monroe 57	-
	58	Monroe 58	-
	59	Monroe 59	-
	60	Monroe 60	-
	61	Monroe 61	-
	62	Monroe 62	-
	63	Monroe 63	-
	64	Monroe 64	-
	65	Monroe 65	-
	66	Monroe 66	-
Newellton 1	67	Newellton-1 67	-
	68	Newellton-1 68	-
	69	Newellton-1 69	-
	70	Newellton-1 70	-
	71	Newellton-1 71	-
	72	Newellton-1 72	-
	73	Newellton-1 73	+
Alexandria	74	Alexandria 74	-
	75	Alexandria 75	-
	76	Alexandria 76	+
	77	Alexandria 77	-
	78	Alexandria 78	-
	79	Alexandria 79	-
	80	Alexandria 80	-

Newellton 2	81	Newellton-2 81	-
	82	Newellton-2 82	-
	83	Newellton-2 83	-
	84	Newellton-2 84	-
	85	Newellton-2 85	-
	86	Newellton-2 86	+
St. Landry	87	St. Landry 87	-
	88	St. Landry 88	-
	89	St. Landry 89	-
	90	St. Landry 90	-
	91	St. Landry 91	-
	92	St. Landry 92	-
	93	St. Landry 93	-
	94	St. Landry 94	-
	95	St. Landry 95	-
	96	St. Landry 96	-
	97	St. Landry 97	-
	98	St. Landry 98	-
	99	St. Landry 99	-
	100	St. Landry 100	-
	101	St. Landry 101	+
Vidalia 1	102	Vidalia-1 102	-
	103	Vidalia-1 103	-
	104	Vidalia- 104	-
	105	Vidalia- 105	-
	106	Vidalia- 106	-
	107	Vidalia- 107	-
Vidalia 2	108	Vidalia-2 108	-
	109	Vidalia-2 109	-
	110	Vidalia-2 110	-
	111	Vidalia-2 111	-
	112	Vidalia-2 112	-
	113	Vidalia-2 113	+
St. Joseph 1	114	St. Joseph-1 114	-
	115	St. Joseph-1 115	-
	116	St. Joseph-1 116	-
	117	St. Joseph-1 117	-
	118	St. Joseph-1 118	-
	119	St. Joseph-1 119	-
	120	St. Joseph-1 120	-
	121	St. Joseph-1 121	-
	122	St. Joseph-1 122	-
St. Joseph 2	123	St. Joseph-2 123	-
	124	St. Joseph-2 124	-

125	St. Joseph-2 125	-
126	St. Joseph-2 126	-
127	St. Joseph-2 127	-
128	St. Joseph-2 128	-
129	St. Joseph-2 129	-
130	St. Joseph-2 130	+

*(+) silicate solubilizer, (-) non-silicate solubilizer

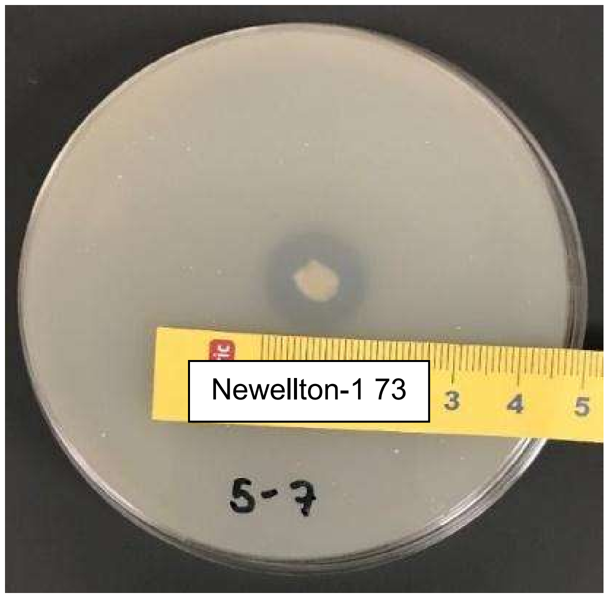


Figure 2.2. Clearing zone around the colony indicating the ability of bacteria to solubilize silicate in silica-amended agar medium.

Silicate-solubilizing bacteria isolates were classified based on their SI demonstrating low (SI < 2.00), intermediate (2.00 < SI < 4.00) and high (SI > 4.0) solubilization capacities (Santi and Goenadi, 2017). It is evident from the results that twenty bacterial isolates produced a zone of solubilization ranging in diameter from 1.17 mm to 2.40 mm. Isolates Winnsboro 54 and St. Joseph-2 130 had the highest solubilization index of 2.4 mm followed by Iowa 46 (2.2 mm) (Table 2.4). Newellton-1 73, St. Gabriel 23 and St. Gabriel 25 produced a clearing zone of 2.14 mm, 1.99 and 1.95, respectively. Raj (1999) reported that SSB release silica (SiO_2) in solution from the water in soluble silicates. Soil contains numerous numbers of bacteria but only few bacteria are found to release Si from natural silicates. Studies have shown that these bacteria solubilize silica aside from releasing other nutrients such as phosphate, K, iron (Fe), and calcium (Ca) from the soil silicate minerals (Santi and Goenadi, 2017).

In the study of Naureen et al. (2015), a total of 111 bacterial strains were isolated from various locations in Pakistan and screened for solubilization of silicate, phosphate, and K on selective media. The highest silicate solubilization zone diameter was 54 mm, observed for bacterial isolate NR-2 (origin, wheat rhizosphere; location, Narowal). It has been reported that bacterial isolates produce extracellular polysaccharides that can accelerate and solubilize insoluble silicate minerals.

2.3.2. *In vitro* Screening of SSB Isolates for Growth-Promoting Activities (GPA)

2.3.2.1. Indole-3-acetic acid production

The 20 SSB isolates were further screened for their abilities of producing IAA. Based on the results, all the twenty SSB produced IAA (Table 2.4). Isolates showed IAA producing ability in liquid culture supplemented with tryptophan in the range of 1.97 to 77.32 ug ml^{-1}. Isolate Bossier 7 produced the highest content of IAA at 77.32 ug ml^{-1}. On the other hand, isolates Newellton-2 86, Alexandria 76 and Vidalia-2 113 produced 34.21, 33.00, and 32.60 ug ml^{-1}, respectively, of IAA. By comparing the IAA amount produced by Bossier 7 and Newellton-2 86, the former isolate produced 52% greater amount of IAA. The appearance of a pink color in the culture broth indicated IAA production which was due to the complex produced by $Fe\text{-}H_2SO_4$ solution and IAA (Aly et al., 2012; Gronemeyer et al., 2012). Ahmad et. al (2005) observed a development of pink color in *Azotobacter* and *Pseudomonas*. Based on their ability to produce IAA, these IAA-producers are potential plant growth-promoter provided that tryptophan, the precursor of IAA, is available and the amount of IAA produced meets the plant requirement (Arshad and Frankenberger, 1990; Marumo, 1986).

The production of IAA can vary among species and strains, and it is also influenced by culture condition, growth stage and substrate availability. As cited by Khan et al. (2016), the greater amount of IAA produced by plant growth-promoting bacteria (PGPB) in LB broth was due to L-tryptophan which was also confirmed from the results of Idris et al. (2007). As cited by Guisain et al. (2015), the ability to produce IAA is considered as an effective tool for screening of growth-promoting microorganisms, as many reports suggested that IAA-producing bacteria have a profound effect on plant growth. They promote lateral and adventitious root formation, which can facilitate high root surface area for nutrient absorption from the soil (Aloni et al., 2006). Also, the production of growth-promoting compounds such as plant enzymes is part of the metabolism of various bacteria associated with plants causing modifications in the morphology of roots (Bashan and Holguin, 1997).

2.3.2.2. Phosphate solubilization

Nine of the 20 SSB were able to dissolve precipitated tricalcium phosphate as shown by clearing zone around isolates grown in Pikovskaya's medium (Table 2.4 and Figure 2.3). Similar observation was reported by Cruz et al. (2014) showing clearing zone around bacterial colony as it dissolved precipitated tricalcium phosphate. Tripti et al. (2012) reported that the zone of formation could be due to the activity of the phosphatase enzyme in bacterial isolates.

Figure 2.3. Phosphate solubilization on Pikovskaya's agar medium by Bossier 8. The clearing zone around the colony indicates the ability to dissolve precipitated tricalcium phosphate.

The principal mechanism for the formation of a clearing zone by the 9 isolates is the secretion of organic acids. The release of organic acids by phosphate-solubilizing bacteria (PSB) dissolves insoluble mineral phosphates (Berthelin et al., 1991). Sarker et al. (2004) have verified this effect on a similar assay, both in solid and culture broth media, which interestingly recorded a decrease in pH in the broth medium used. The secretion of organic acids by PSB have also been well-documented by Rodriguez and Fraga (1999). In the present study, PSI of the isolates ranged from 1.14 to 4.50 (Table 2.4). Highest efficiency was exhibited by Bossier 8 isolate. These PSB could serve as efficient biofertilizer candidates for improving the N and P nutrition of crops. In crop production, PSB are also used as inoculants. The use of these bacteria as bio-inoculants simultaneously improves P uptake and crop yield of the plant. Among the most powerful phosphate-solubilizers are the strains of the genera *Pseudomonas*, *Bacillus* and *Rhizobium*. Phosphatases play a major role in the mineralization of organic P in soil making it available for plant uptake (Rodriguez and Fraga, 1999).

2.3.2.3. 1-aminocyclopropane-1-carboxylic acid-deaminase activity

Of the twenty SSB screened, only nine were positive for ACC deaminase activity as indicated by their growth on the Dworkin and Foster's minimal salts agar medium amended with ACC (DF-ACC agar) (Figure 2.4). According to El-Tarabily (2008), these bacterial isolates both utilize ACC and produce ACC deaminase (El-Tarabily, 2008). The production of ACC deaminase by plant growth-promoting rhizobacteria (PGPR) reduces the stress inducible ethylene level in host plants (Karthikeyan et al., 2012).

The enzyme ACC deaminase breaks down ACC, an intermediate biosynthetic precursor of the hormone ethylene in plant tissues, to ammonia and α-ketobutyrate (Glick et al., 2007). As a result, the production of ethylene is reduced. This mechanism is evident in several rhizospheric bacteria. Production of ACC deaminase hydrolytic enzyme can be useful tool to mitigate plant stress caused by adverse environmental stresses, as this reduces the stress inducible ethylene level in host plants (Singh and Jha, 2015). A study by Glick et al. (2007) proved this claim where bacteria positive of ACC deaminase activity reduced the level of stress ethylene, conferring resistance and resulting in better plant growth under various stresses such as salt, flooding, heavy metal, and diseases. In crop production, ACC deaminase-containing PGPR are of great significance. These bacteria are valuable to plant growth as plants are often subjected to ethylene-producing stress. Thus, by lowering level of ACC in the stressed plant, the amount of stress ethylene synthesis is also limited as well as damage to the plant (Ali et al., 2013).

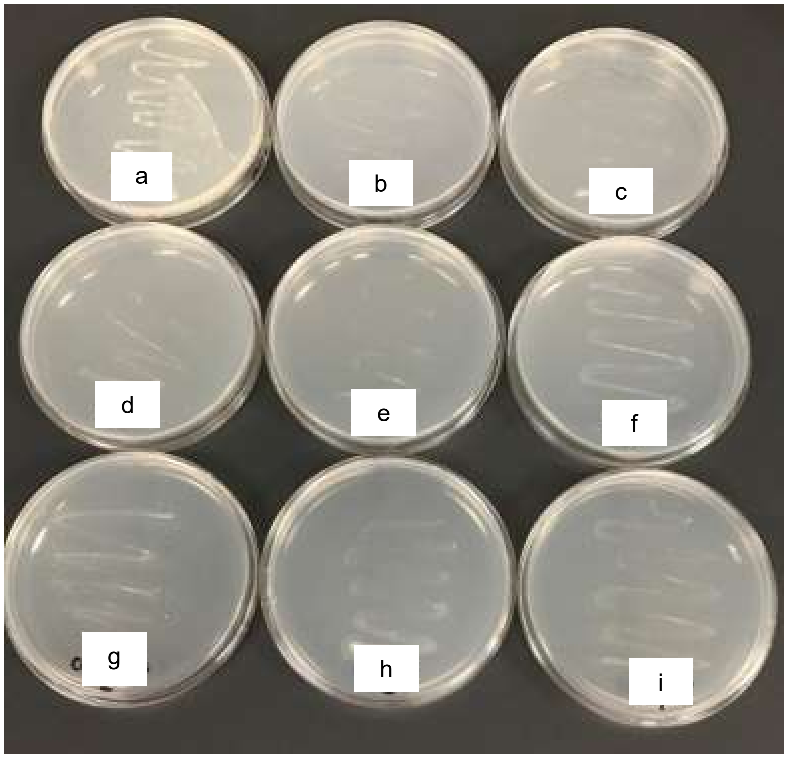


Figure 2.4. Silicate-solubilizing bacteria positive for ACC deaminase production: (a) Winnsboro 54, (b) St. Joseph-2 130, (c) Newellton-1 73, (d) Iowa 46, (e) Iowa 44, (f) St. Landry 101, (g) Crowley 20, (h) Bossier 10 and (i) Bossier 8. The growth on DF agar media indicates production of ACC deaminase.

2.3.2.4. Nitrogen fixation

Only 4 of the 20 SSB isolates were potential N-fixers as indicated by their growth on Jensen's medium (N-free medium). This suggests that these bacterial isolates can produce the enzyme nitrogenase. All N-fixing bacteria use a highly conserved enzyme complex called nitrogenase, which is responsible for the conversion of dinitrogen (N_2) to ammonia (NH_3). There are three types of nitrogenases; the first nitrogenase consists of a molybdenum (Mo)-Fe and an Fe protein, the second is a vanadium (V)-Fe protein and an Fe protein, and the third nitrogenase does not appear to contain either Mo, or V. Aerobic organisms face special challenges to N fixation when oxygen reacts with the iron component of the proteins making nitrogenase inactive (NRC, 1994).

Hussain and Srinivas (2013) used Jensen's medium for the isolation of N-fixing bacteria such as *Azotobact*er from the rhizosphere soil of two agroforestry tree species. Nitrogen-fixing bacteria will not compete with other non-N-fixing soil microbes for nutrients on the nutrient agar medium but will have an advantage on the selective N-free mineral agar medium. Colonies of N-fixing bacteria will grow well on the N-deficient medium (Health and Safety Checked, 2008). In addition, free-living diazotrophs have been reported to improve nutrient uptake efficiency and to fix N_2 through associative and endophytic associations with graminaceous plants. Nitrogen fixation and N-use efficiency have a significant role because of its importance in sustainable agriculture, especially in cropping systems involving rotations of rice and legumes (Raja et al., 2006).

Table 2.4. Silicate solubilization and production of other growth-promoting compounds by the twenty bacterial isolates.

	Silicate Solubilization		Other growth-promoting activities			
Isolate	Silicate Solubilization (after 7 DAI) (mm)	Solubilization Index (SI)[a]	Indole-3-acetic acid Production (after 24 hours) (ug ml^{-1})	Phosphorus Solubilization Index (mm)	ACC Deaminase Activity[b]	Nitrogen Fixation[c]
St. Gabriel 23	1.99	Low	17.90	0	-	+
St. Gabriel 24	1.88	Low	15.20	0	-	-
St. Gabriel 25	1.95	Low	14.57	0	-	+
St. Gabriel 26	1.8	Low	1.97	0	-	-
Bossier 7	1.6	Low	77.32	0	-	-
Bossier 8	1.36	Low	5.25	4.5	+	-
Bossier 9	1.4	Low	15.20	1.38	-	-
Bossier 10	1.36	Low	7.87	0	+	-
Crowley 19	1.17	Low	8.57	0	-	-
Crowley 20	1.2	Low	17.20	0	+	-
Iowa 46	2.2	Intermediate	2.15	0	+	+
Winnsboro 54	2.4	Intermediate	14.21	2.33	+	-
Newellton-1 73	2.14	Intermediate	12.49	2.83	+	-
Alexandria 76	1.17	Low	33.00	1.43	-	-
St. Landry 101	1.25	Low	16.23	0	+	+
Newellton-2 86	1.8	Low	34.21	1.6	-	-
St. Joseph-2 130	2.4	Intermediate	15.55	1.14	+	-
Iowa 44	1.2	Low	24.21	1.6	+	-
Vidalia-2 113	1.2	Low	32.60	1.33	-	-
Iowa 47	1.17	Low	7.77	0	-	-

[a]Silicate-solubilizing bacteria isolates were classified based on their SI as demonstrating low (SI < 2.00), intermediate (2.00 < SI < 4.00) and high (SI > 4.0) solubilization capacities (Santi and Goenadi, 2017).

[b]ACC Deaminase Activity: (+) ACC deaminase-producer, (-) non-ACC deaminase-producer

[c]Nitrogen Fixation: (+) Nitrogen-fixer, (-) Non-nitrogen fixer

2.3.4. Identification of Silicate-Solubilizing Bacterial Isolates

2.3.4.1. Morphological Identification

Gram staining was conducted to determine if the isolates are Gram positive or Gram negative (Table 2.5 and Figure 2.5). All selected isolates were found to be Gram negative except for isolates Crowley 20, Iowa 46, Iowa 47 and Newellton-2 86. Gram-positive cells have a thick peptidoglycan layer and stain blue to purple. On the other hand, Gram-negative cells have a thin peptidoglycan layer and stain red to pink (Smith and Hussey, 2012). Gram staining is a very important preliminary step in the initial characterization and classification of bacteria. Differentiation of bacteria into gram positive and gram negative is the basic foundation on which bacterial identification is built. It differentiates bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls (Rao, 2006).

The sporulating Gram-positive bacteria suggest biological solutions to the formulation problems that have plagued biocontrol. Sporulating Gram-positive microorganisms, such as *Bacillus* and *Streptomyces*, offer heat- and desiccation-resistant spores, which can be formulated readily into steady products (Emmert and Handelsman, 1999). Gram-positive bacteria are a good source of antibiotics (Nandi, 2004). These spore products can be formulated as a dry powder, while Gram-negative microorganisms, like *Pseudomonas syringae*, are formulated as frozen cell pellets that must be kept on dry ice until application.

The Gram-negative characteristic is common among N-fixing bacteria. Dhevendaran et al. (2013) isolated and identified N-fixing bacterial strains from medicinal plants which were all Gram-negative. In rice rhizosphere, Islam et al. (2012) also reported all Gram-negative N-fixing bacterial strains with maximum number belonged to genus *Burkholderia*. Some Gram-negative bacteria such as Genus *Enterobacter* are known to have a wide range of plant-growth-promoting (PGP) characteristics such as N fixation, soil P solubilization, ACC deaminase, siderophore and antibiotics production, and in the enhancement of soil porosity. Numerous *Enterobacter* strains have these characteristics which promote plant growth and suppress soil-borne plant pathogens. These PGP abilities of *Enterobacter* can make them a potential bio-inoculant candidate suitable for plant growth and development (Jha et al., 2011).

In this study, majority of the SSB isolates are rod in shape: St. Gabriel 24, St. Gabriel 25, St. Gabriel 26, Bossier 7, Bossier 8, Bossier 9, Bossier 10, Crowley 19, Crowley 20, Iowa 46, Winnsboro 54, and St. Landry 101 (Figure 2.5) but there are also few isolates that are coccoid in shape. Related studies of Jha et al. (2011) characterized *Enterobacter* as straight rod, motile with peritrichous flagella and are facultatively anaerobic.

Colony colors of the twenty SSB are as follows: Crowley 20, Crowley 19, Bossier 9, St. Gabriel 23 and Newellton-1 73 were all off-white; Iowa 47, creamy white; Iowa 46, yellow; Bossier 7, Bossier 8, Bossier 10, St. Gabriel 26, St. Gabriel 24, St. Gabriel 25, Winnsboro 54, Iowa 44, St. Joseph-2 130 and Vidalia-2 113 were yellowish-brown, and Alexandria 76, St. Landry 101, Newellton-2 86 were light brown. The size of the twenty SSB ranged from 0.55 to 2.75 µm (Table 2.5 and Figure 2.5). Genus *Enterobacter* has a size of 0.6-1.0 micrometers by 1.2-3.0 micrometers (Holt, 1994). *Bacillus* about average size is 1.1 to 1.5 µm wide by 2.0 to 6.0 µm long. Comparing the sizes of an unidentified bacterium from a known bacterium is also useful in the initial characterization of the microorganism.

Table 2.5. Morphological characteristics of twenty silicate-solubilizing bacterial isolates.

Isolates	Size (um)	Gram Staining	Shape
St. Gabriel 23	1.10	-	Coccoid
St. Gabriel 24	0.83	-	Rod
St. Gabriel 25	1.10	-	Rod
St. Gabriel 26	1.10	-	Rod
Bossier 7	1.10	-	Rod
Bossier 8	1.65	-	Rod
Bossier 9	1.10	-	Rod
Bossier 10	1.10	-	Rod
Crowley 19	0.83	-	Rod
Crowley 20	2.75	+	Rod
Iowa 46	1.65	+	Rod
Winnsboro 54	1.10	-	Rod
Newellton-1 73	0.83	-	Coccoid
Alexandria 76	0.28	-	Coccoid
St. Landry 101	1.10	-	Rod
Newellton-2 86	1.10	+	Coccoid
St. Joseph-2 130	1.10	-	Coccoid
Iowa 44	0.55	-	Coccoid
Vidalia-2 113	0.83	-	Coccoid
Iowa 47	1.10	+	Coccoid

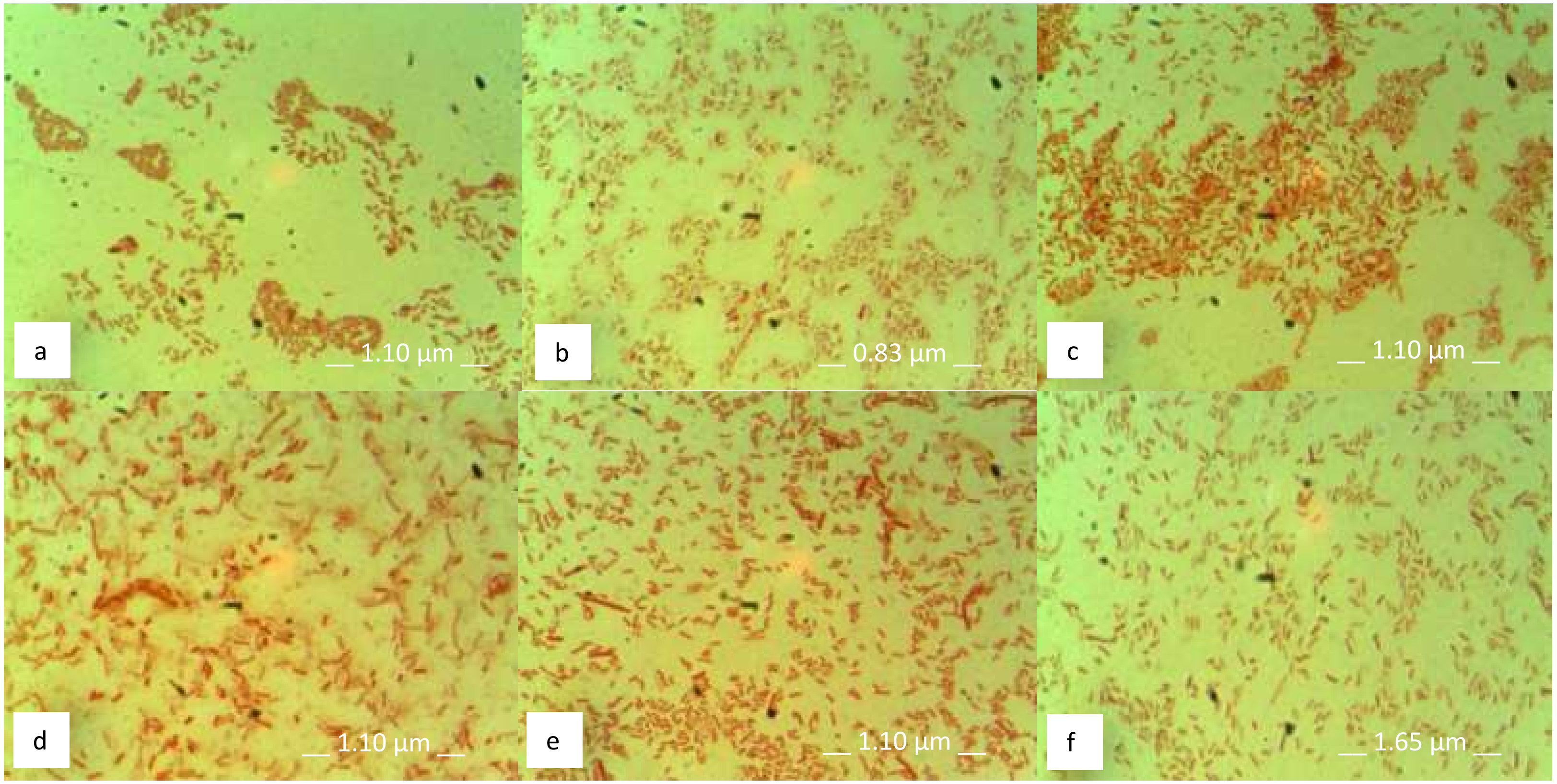
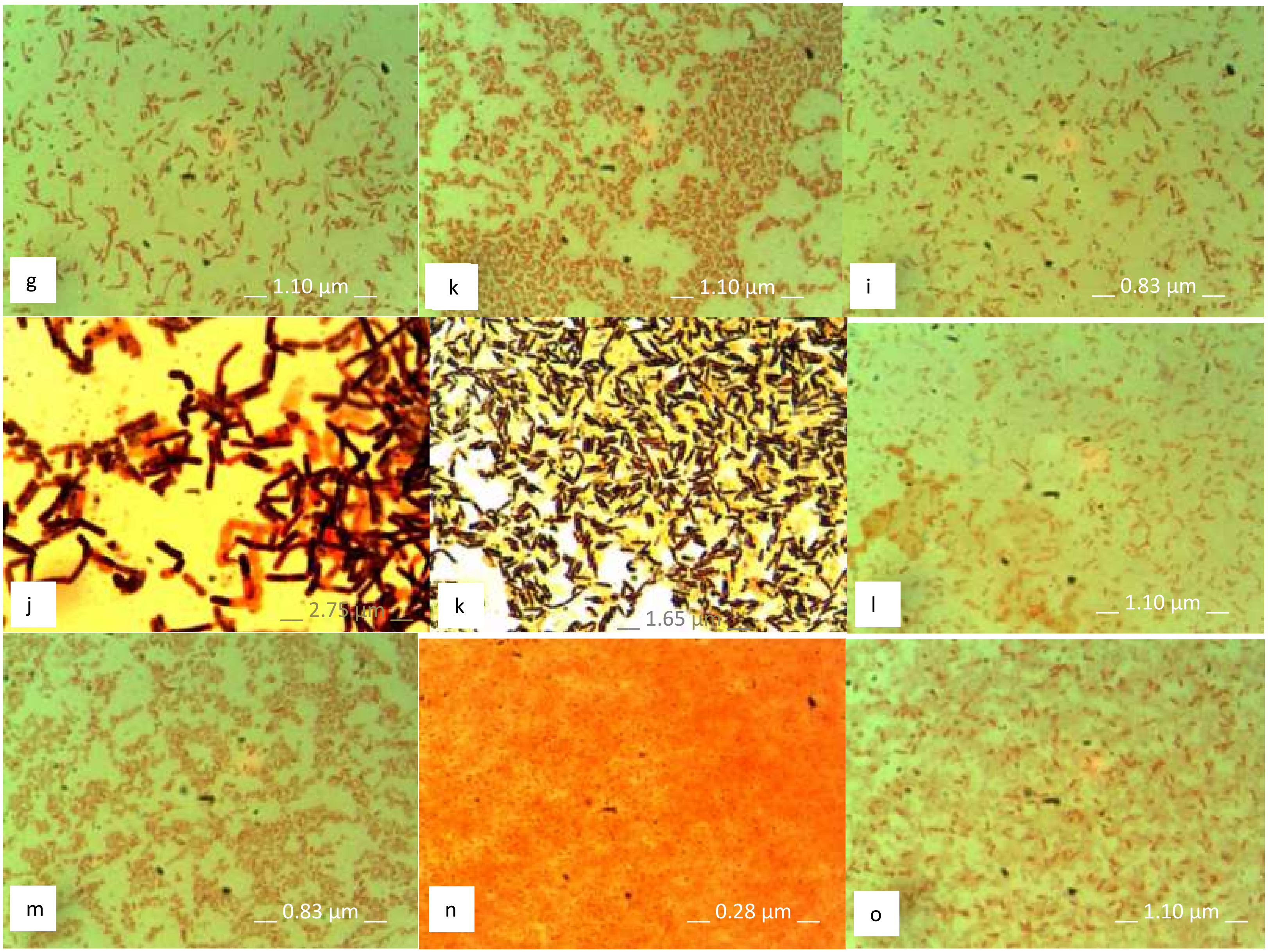


Figure 2.5. Gram staining of the twenty silicate-solubilizing bacteria (a) St. Gabriel 23, (b) St. Gabriel 24, (c) St. Gabriel 25, (d) St. Gabriel 26, (e) Bossier 7, (f) Bossier 8, (g) Bossier 9, (h) Bossier 10, (i) Crowley 19, (j) Crowley 20, (k) Iowa 46, (l) Winnsboro 54, (m) Newellton-1 73, (n) Alexandria 76, (o) St. Landry 101, (p) Newellton-2 86, (q) St. Joseph-2 130, (r) Iowa 44, (s) Vidalia-2 113, and (t) Iowa 47. (Magnification: 1,250x)
(fig. cont'd.)


g
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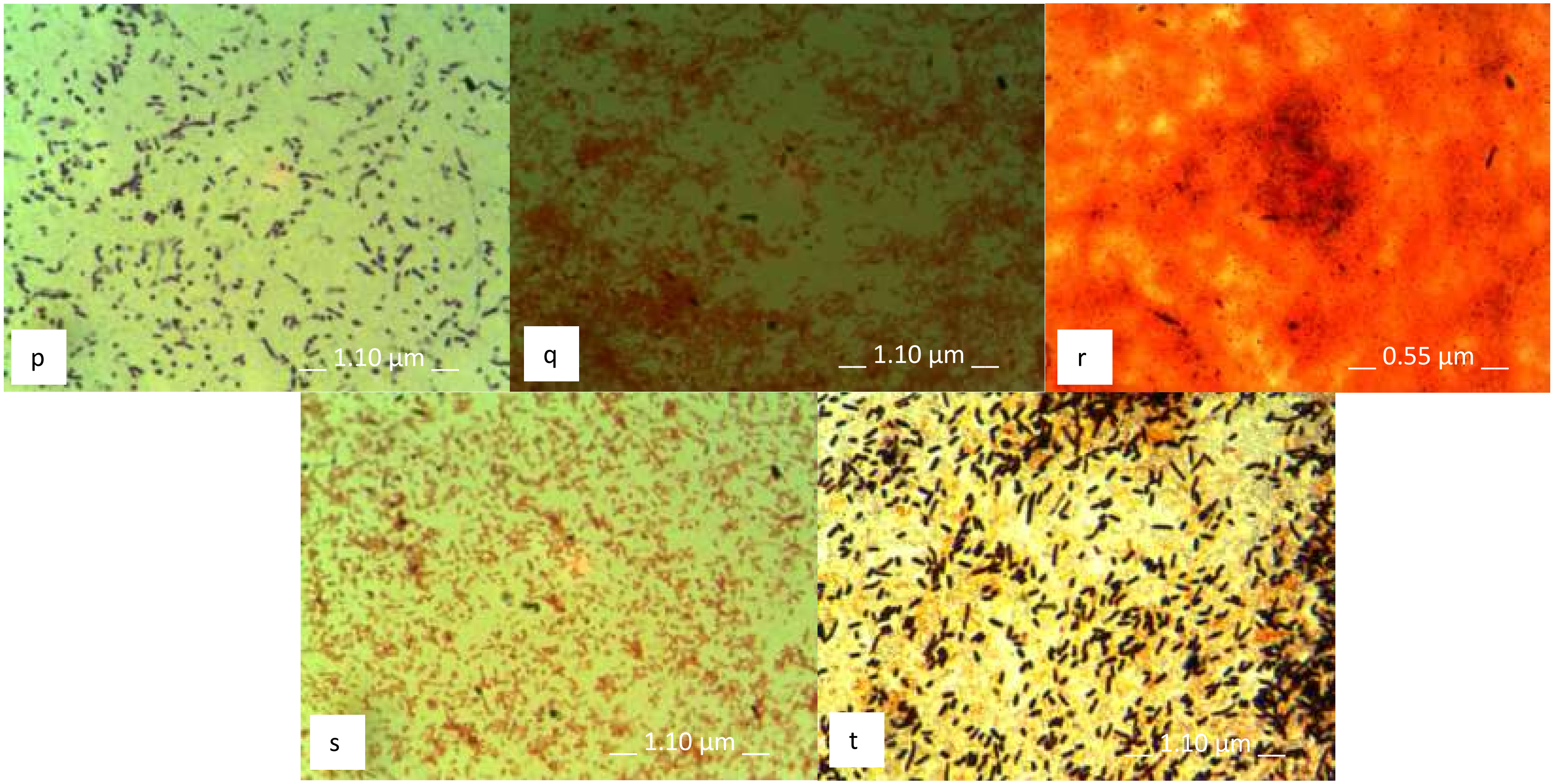
(fig. cont'd.)


p
1.10 µm
q
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r
0.55 µm
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1.10 µm
t
1.10 µm

2.3.4.2. Molecular Identification using 16S Ribosomal DNA Analysis

In this study, the amplification of an expected 1.5 Kb fragment was observed in all twenty samples. Figure 2.6 shows the PCR products in stained using ethidium bromide. Lanes 1, 7, 14 and 27, 1kb+ ladder; Lanes 6, 19 and 26, positive control; Lane 2, St. Gabriel 23; Lane 3, St. Gabriel 24; Lane 4, St. Gabriel 25; Lane 5, St. Gabriel 26; Lane 8, Bossier 7; Lane 9, Bossier 8; Lane 10, Bossier 9; Lane 11, Bossier 10; Lane 12, Crowley 19; Lane 13, Crowley 20; Lane 15, Iowa 46; Lane 16, Winnsboro 54; Lane 17, Newellton-1 73; Lane 18, Alexandria 76; Lane 20, St. Landry 101; Lane 21, Newellton-2 86; Lane 22, St. Joseph-2 130; Lane 23, Iowa 44; Lane 24, Vidalia-2 113; and Lane 25, Iowa 47. The polymerase chain reaction products produced the expected band size of approximately 1, 500 bp.

Based on the sequencing information (Table 2.6) and the phylogenetic tree (Figure 2.7), the putative SSB isolates were identified into four genera: *Aeromonas, Bacillus, Enterobacter* and *Pseudomonas*. The phylogenetic tree shows that the probable classification of St. Gabriel 23, St. Gabriel 24, St. Gabriel 25, St. Gabriel 26, Bossier 8, Bossier 9, Bossier 10, Crowley 19, Winnsboro 54, Newellton-1 73, St. Landry 101, St. Joseph-2 130 is genus *Pseudomonas* (Figure 2.6 and Table 2.6). On the other hand, the following isolates: Crowley 20, Iowa 46, Vidalia-2 113, and Iowa 47 were classified under genus *Bacillus* based on the phylogenetic tree.

In the study of Vasanthi et al. (2018), silicate solubilization was observed both in Gram-negative *Pseudomonas* and Gram-positive *Bacillus* indicating that both Gram-positive and Gram-negative organisms are involved in silicate solubilization in soil and other natural environments. The study of Liu et al. (2006) showed that *Bacillus mucilaginosus* disintegrated minerals and insoluble silicic acid by releasing K ion and Si in the form of monosilicic acid to form organic acids and polysaccharides. Organic acids establish organic ligands and enhance silicate dissolution through the formation of destabilizing-framework surface complexes and metal complexation.

Based on the molecular analysis conducted, the probable classification of isolate Bossier 7 was genus *Enterobacter*. *Enterobacter* is a very diverse genus of bacteria and also feasible for its potential use in agriculture as a bio-inoculant. Lee et al. (2019) showed that *Enterobacter ludwigii* GAK2 could be useful in regulating the levels of phytohormones and ammonia production as well as Si and P solubilization. Similarly, *E. ludwigii* GAK2 could produce organic acids such as citric acid, lactic acid, and acetic acid. Therefore, the higher Si content of the bacteria treated plants can possibly be due to the organic acid produced by the microorganisms that solubilized the insoluble metal and, hence, increased the uptake. This is consistent with the results of Vyas and Gulati (2009) where bacteria like *Pseudomonas* produced the organic acid like oxalic, malic, lactic, 2-ketogluconic, formic, succinic, and citric acids which improved the growth of maize.

The probable identity of isolates Iowa 44, Vidalia-2 113, Newellton-2 86 is genus *Aeromonas* based on molecular analysis. In the study of Santi et al. (2018), *Aeromonas* is one of the major components of BioSilAc (Biosilica fertilizer). In the study of Santi and Goenadi (2017), silicate-solubilizing activities by SSB were determined using inductively coupled plasma-atomic emission spectrometry. *Aeromonas punctata* RJM 3020, *Burkholderia cenocepacia* KTG, and *B. vietnamiensis* ZEO3 have been shown to improve the solubilization of SiO_2 originated from quartz.

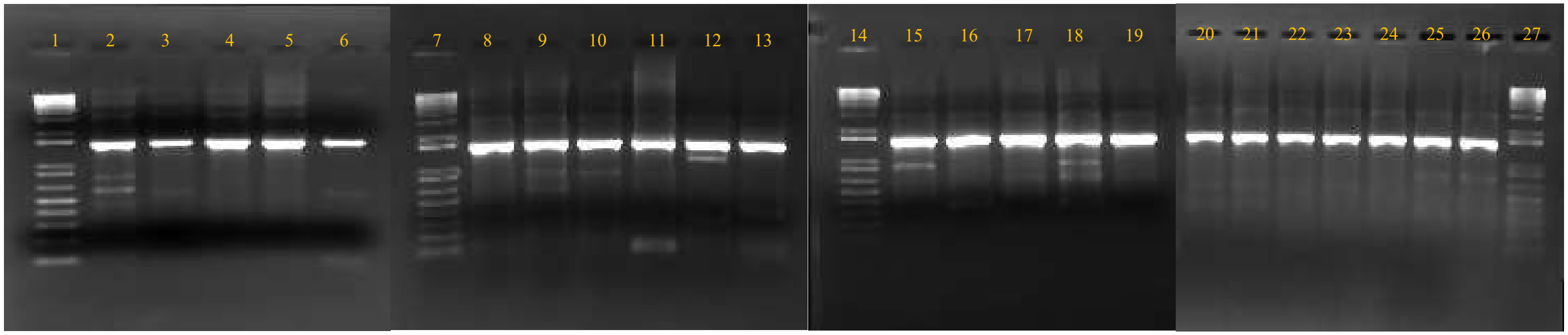


Figure 2.6. PCR products of twenty silicate-solubilizing bacteria with approximately 1.5 Kb length using 308F and 312R primers ran in 1% agarose gel and viewed under UV using UVP Doc It® gel documentation system (UVP, LLC, Upland, California, USA) after staining with ethidium bromide. Lanes 1, 7, 14 and 27, 1kb+ ladder; Lanes 6, 19 and 26, positive control; Lane 2, St. Gabriel 23; Lane 3, St. Gabriel 24; Lane 4, St. Gabriel 25; Lane 5, St. Gabriel 26; Lane 8, Bossier 7; Lane 9, Bossier 8; Lane 10, Bossier 9; Lane 11, Bossier 10; Lane 12, Crowley 19; Lane 13, Crowley 20; Lane 15, Iowa 46; Lane 16, Winnsboro 54; Lane 17, Newellton-1 73; Lane 18, Alexandria 76; Lane 20, St. Landry 101; Lane 21, Newellton-2 86; Lane 22, ST. Joseph-2 130; Lane 23, Iowa 44; Lane 24, Vidalia-2 113; and Lane 25, Iowa 47.

Table 2.6. Molecular identification of twenty silicate-solubilizing bacterial isolates.

Isolates	Probable Identity	% Similarity
St. Gabriel 23	*Pseudomonas cremoricolorata* DSM 17059T *Pseudomonas monteilii* BD18-R15T	99
St. Gabriel 24	*Pseudomonas frederiksbergensis* SS17T	99
St. Gabriel 25	*Pseudomonas* sp.	96
St. Gabriel 26	*Pseudomonas teessidea* PR65T	100
Bossier 7	*Enterobacter ludwigii* HE15T	99
Bossier 8	*Pseudomonas moorei* MY-A1T	100
Bossier 9	*Pseudomonas stutzeri DSM 5190T*	100
Bossier 10	*Pseudomonas baetica a390*	100
Crowley 19	*Pseudomonas teessidea* B26T	99
Crowley 20	*Bacillus ginsengisoli* SX7T	99
Iowa 46	*Bacillus marisflavi* TF-11	99
Winnsboro 54	*Pseudomonas frederiksbergensis* SS17T	99
Newellton-1 73	*Pseudomonas koreensis* Ps 9-14T	100
Alexandria 76	*Aeromonas jandaei* ATCC 49568T	100
St. Landry 101	*Pseudomonas moraviensis* F1-2-13T *Pseudomonas Teessidea* SCAU601T	99
Newellton-2 86	*Aeromonas* sp.	99
St. Joseph-2 130	*Pseudomonas plecoglossicida* FPC951T	100
Iowa 44	*Aeromonas encheleia* CECT 4342T	100
Vidalia-2 113	*Aeromonas encheleia* CECT 4342T	99
Iowa 47	*Bacillus pumilus* ATCC 7061T *Bacillus pumilus* NBRC 12092T	99

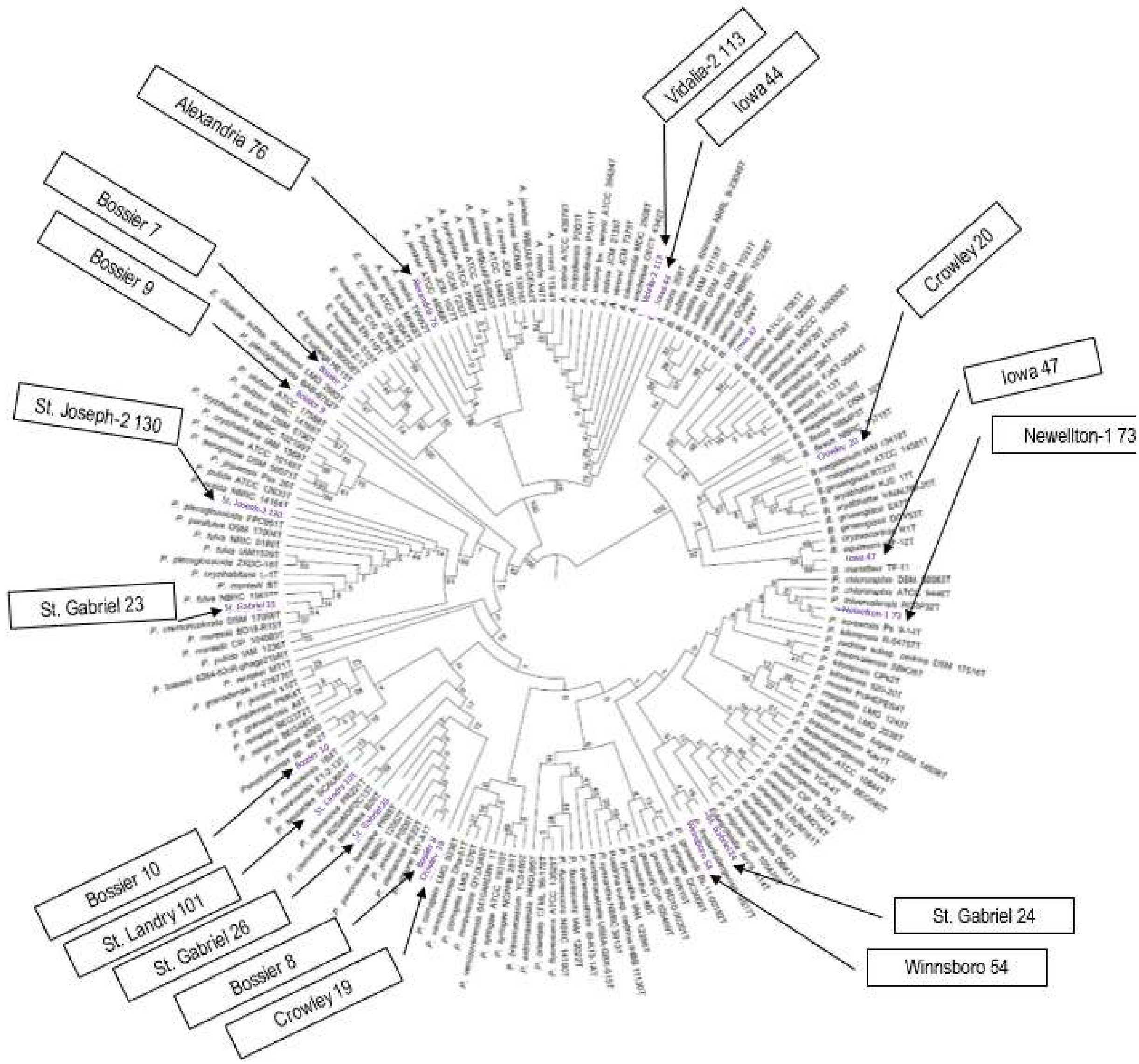


Figure 2.7. Phylogenetic tree of twenty silicate-solubilizing bacteria. (See Table 2.6 for the complete name).

2.4. Conclusions

Excessive use of chemical fertilizers may negatively affect overall soil health and the environment. There is a need to develop alternative strategies to ensure competitive yields of crops and at the same time maintain soil ecological balance. The use of soil microorganisms as microbial inoculants in agriculture is considered an alternative approach to enhance crop growth.

Silicate-solubilizing bacteria isolated from Louisiana soils proved to produce multiple plant growth-promoting compounds such as phosphatase, nitrogenase, ACC deaminase, and indole-3-acetic acid enzyme. Overall, the results of this research present a practical approach to improve the bioavailability of Si in Louisiana soils through strategic cultural management practices that favor the growth and activity of SSB. This research is more valuable to crops which have a high demand for Si like sugarcane that removes about 181kg Si per ton of stalk which is significantly higher than the removal rate for N and K. There are researchers working on Si at the soil fertility level but few researches on biological aspects are being conducted. In addition, environmental concerns and rising cost of chemical fertilizers are some major concerns in crop production. Hence, alternative technologies like utilizing beneficial microorganisms like SSB should be implemented. This would offer the industry a practical, innovative, and ecologically-smart crop care solution, not to mention its huge potential as a commercial product.

However, bacterial isolates in this study still have to be evaluated on a plant-soil system in order to uncover their efficacy as effective microbial inoculants. Most importantly, the molecular mechanism behind these growth-promoting activities should be elucidated under all these circumstances.

Chapter 3: Effect of Silica-Solubilizing Bacteria Inoculants on Plant-Available Silicon Content of Selected Louisiana Soils and Its Impact on Rice Productivity

3.1. Introduction

Louisiana growers produce rice (*Oryza sativa* L.), sugarcane (*Saccharum officinarum*) and wheat (*Triticum aestivum)*, all of which have a high demand for silicon (Si). A survey of the status of Si in rice grown in the southwestern region of Louisiana revealed that in over 60 percent of the fields investigated, the Si content in harvested straw was consistently lower than the minimum sufficiency level of 50 mg kg^{-1} (Kraska and Breitenbreck, 2010). Significant increases in the relative biomass yield of rice in five out of six soil series to which Si was applied were reported in a greenhouse study investigating rice response to Si fertilization in various soil series collected across the state (Babu et al., 2016), with the highest relative biomass yield reported in Commercial silt loam soils.

Silicon is an important nutrient for the safe and competitive growth of all Asian cereals, including rice (Brunings et al., 2009). The role of Si in plant health and growth in Si accumulating crops was investigated and it appeared to be significantly effective (Jinab et al., 2008). Research shows that adequate Si uptake can boost the tolerance of agronomic crops, especially rice, to both abiotic and biotic stress (Ma and Takahashi, 2002). Although Si is not considered an essential element for higher plants, many plant species, particularly tropical graminaceous plants such as rice, which is a hyper-Si accumulator, have been shown to benefit from Si through healthy growth and development (Liang et al., 2007; Lee et al., 2019).

In the study conducted by Babu et al. (2016), the highest grain yield was observed in rice grown on wollastonite-treated soils. Grain yield increased was 16.5% attributed to wollastonite application at 680 kg Si ha^{-1} in Sharkey clay. Another study by Agostinho et al. (2017)

demonstrated that wollastonite and slag application in rice were effective in raising Si content. Silicon, considering its abundance in the earth's crust, is mainly found in insoluble forms that are not readily available for plant uptake. Until solubilized by the weathering action of rocks or biological activity of plant roots and microorganisms, it remains in insoluble forms (Naureen et al., 2015).

The major agents that contribute to mineral weathering are microorganisms. In order to solubilize biotite, which contains significant amounts of silicate minerals, the action of microorganisms such as Proteobacteria, Aminobacter, Burkholderia, Collimonas, Janthinobacterium, Dyella, and Frateuria is required (Lee et al., 2019). Those bacteria that can solubilize silica are called silica-solubilizing bacteria (SSB). These bacteria play an effective role in solubilizing insoluble silicate which contributes to soil fertility and enhances plant defense mechanisms. Silicate solubilizing bacteria are found in soil, water, marine sediments, and silicate minerals, but their number is lower than the overall bacteria, demonstrating their uniqueness (Vasanthi et al., 2018; Naureen et al., 2015).

The reduction in paddy yields is not affordable for agricultural system under changing socio-economic conditions around the world. Major nutrients (nitrogen [N], phosphorus [P], and potassium [K]) are already at an optimum level in practice, but the yield gap is still present, so it is important to include nutrients such as Si in rice production system. The SSB community is one of the main contributors to the availability of Si. The role of SSB to Si's availability has not been elucidated. In the light of the above-mentioned discussions, the present study was intended to investigate the effect of SSB on the absorption of Si and the productivity of rice using various carriers on two Louisiana soils.

Specifically, the objectives of the study are the following:

a. Quantify changes on plant-available Si content of soils inoculated with SSB

b. Evaluate yield response of rice grown on soils inoculated with SSB and fertilized with wollastonite

3.2. Materials and Methods

The study involved collection of soil samples (S1, S2), chemical characterization of soils, greenhouse experiment; collection, handling, and management of greenhouse and analytical data; and, scanning electron microscopy analysis, methyl blue colorimetric test, and interpretation of data.

3.2.1. Description of Treatments

Two soils were selected on the basis of Si content: Gigger silt loam (low Si, <40 mg Kg^{-1}) and Commerce silt loam (medium Si, 40-100 mg Kg^{-1}). The study had 8 treatment combinations of the three factors given below:

Soil (S)

S1 = Gigger silt loam (low Si, <40 mg Kg^{-1})

S2 = Commerce silt loam (medium Si, 40-100 mg Kg^{-1})

Rates of Wollastonite Fertilization (F)

F1 = no wollastonite

F2 = with wollastonite (500 Kg $Siha^{-1}$)

Rates of SSB Inoculation (I)

I1 = uninoculated

I2 = inoculated

The 2 x 2 x 2 complete factorial treatment structure was arranged in a randomized complete block design (RCBD) with 6 replicates.

3.2.2. Collection and Processing of Bulk Soil Samples

Soils were collected from St. Gabriel (soil classification- Commerce silt loam (sl), Fine-silty, mixed, superactive, nonacid, thermic Fluvaquentic Endoaquepts; coordinates- 30.2625, -91.09722) and Winnsboro (soil classification- Gigger silt loam (sl), Fine-silty, mixed, active, thermic Typic Fragiudalfs; coordinates- 32.1418, -91.6862). It is imperative that cross contamination is prevented during the transport and processing of bulk soil samples. Soil was sieved through a 5 mm screen, homogenized, and allowed to air-dry. Composite samples were also taken from each processed bulk soil for initial chemical characterization. The chemical characteristics of the soil used in this study were given in Table 3.1.

For the potting of soils, enclosed pots with 11-L capacity were filled with about 15 kg air-dried, sieved soil. Each pot was labelled corresponding to a treatment combination.

3.2.3. Chemical characterization of soils after harvest

Soils were analyzed after for the following: pH (1:1, soil:water suspension), 0.5 M acetic acid extractable Si, and Mehlich-3 extractable nutrients.

3.2.4. Seed Surface Sterilization

Mermentau rice seeds were washed with tap water five times to eliminate unwanted particles then soaked in 95% ethyl alcohol for 2.5 minutes and washed with sterile distilled water five times. The seeds were then soaked in 30% bleach for 30 seconds and washed again with sterile distilled water five times to rinse off the 30% bleach.

3.2.5. Preparation of Inoculant Carriers and SSB Inoculants

The following inoculant carriers were used: slag, bagasse, and rice hull. One hundred grams (g) of each individual carrier was weighed and packed into autoclavable plastic bags and sterilized for 1 hour at 121°C for three consecutive days.

Actively growing SSB was inoculated into 100 mL tryptic soy broth (TSB) and was incubated for 24 hours at room temperature (28-30 °C). After incubation, a certain amount of the culture broths was aseptically inoculated into the 100 g sterilized inoculant carriers which brought each inoculant carrier moisture to approximately field capacity (Appendix Table 3.1). The SSB inoculants were incubated for one week at room temperature.

3.2.6. Inoculant Application and Planting

The surface sterilized seeds were coated with SSB using the different inoculant carriers (slag, bagasse, and rice hull) for 30 minutes. Five seeds were sown at about 2.5 cm deep in each pot.

3.2.7. Fertilizer Application

Urea (267 kg ha^{-1}) as N source was applied based on LSU AgCenter fertilizer recommendation for rice. At 2 to 3 leaf stage, urea granules were mixed thoroughly on the upper 15 cm of the potted soil followed by immediate flooding.

Table 3.1. Chemical characteristics of the soil used.

Soil	pH (1:1 Water)	Ca	Cu	Mg	P	K	Na	S	Zn
		---mg kg^{-1}---							
St. Gabriel	7.970	2,772.42	3.21	412.06	36.99	117.36	62.56	9.15	4.06
Winnsboro	5.080	736.53	1.52	211.21	16.54	150.33	217.53	17.21	7.65

3.2.8. Data

A quantitative measure of the plant dry biomass, plant Si content and uptake (shoot Si, root Si, and grain Si), grain yield, soil pH, and soil and nutrient uptake were evaluated for two consecutive years. Agronomic data and plant nutrient uptake were all expressed in g m^{-2}. Nutrient uptake was computed by multiplying the percent nutrient content of the plants to the corresponding dry weight (straw, roots, grains, etc.).

Microscopic characterization of Si deposition was also determined using Scanning Electron Microscopy and EDX microanalysis and mapping during the rice late vegetative-early reproductive stage.

3.2.9. Plant Nutrient Analysis

3.2.9.1. Silicon analysis

Silicon content in plant tissue samples was determined by the Oven-Induced Digestion procedure (OID) (Kraska and Breitenbeck, 2010) followed by the Molybdenum Blue Colorimetric (MBC) procedure (Hallmark et al., 1982). In order to take out any remaining moisture from the samples, one hundred milligrams of ground tissue samples were weighed into 50-mL polyethylene centrifuge tubes and oven-dried at 60°C for 15 minutes. Before putting it back in the oven at 95°C for 30 minutes, five drops of octyl alcohol and 2 mL of hydrogen peroxide (H_2O_2) were added to the tubes. Samples were then taken from the oven and 4 mL of 50% sodium hydroxide (NaOH) was added. Tubes were loosely capped and placed back into the oven. Every 15 minutes for 4 hours, tubes were taken out of the oven and gently mixed using a vortex mixer. After 4 hours, 1 mL of ammonium fluoride (NH_4F) was added to the digested samples, mixed, and diluted to a final volume of 50 mL distilled water.

For the MBC procedure, 2 mL aliquot of plant digest solution was obtained and placed into a 30-mL centrifuge tube. Then, 10 mL of 20% acetic acid and 2 mL of 0.26 M ammonium molybdate [$(NH_4)_6Mo_7O_2$] were added. Samples were then allowed to stand for 5 minutes before adding 2 mL of 20% tartaric acid. The sample solution was mixed and allowed to sit for 2 minutes before adding 2 mL of ANSA (reducing agent composed of 0.5 mg of 1-amino-2-naphthol-4-sulphonic acid, 1.0 g of sodium sulfite and 30.0 g of sodium bisulfite). The samples were diluted with 20% acetic acid to a final volume of 30 mL, and absorbance readings were measured at 630 nm using UV-Visible Spectrophotometer (Hach DR 500). Standard series at rates of 0, 0.4, 0.8, 1.6, 3.2, 4.8, 6.4, and 9.6 ug mL^{-1} of Si, as well as references and blanks samples were also measured.

3.2.9.2. Multi-element analysis

For essential nutrient contents, plant tissue samples were digested with concentrated nitric acid (HNO_3) and 30% H_2O_2 at 152°C and analyzed using inductively coupled plasma (ICP) –Optical Emission Spectroscopy (OEM). Five hundred milligrams of ground plant tissue samples were weighed and placed into a 125-mL digestion tube and 5 mL of concentrated HNO_3 was added. Each sample was mixed using a vortex mixer, and after 50 minutes the tubes were set on the heating block for five minutes at 152°C to initiate vigorous boiling. The tubes were removed from the digestion block and allowed to cool down for 15 minutes before adding 3 mL of 30% H_2O_2. Small glass funnels were placed on each tube to prevent excessive evaporation and drying of solution while digesting. Samples were returned to the heating block and allowed to digest for 2 hours and 45 minutes. Digested samples were allowed to cool down overnight, then were mixed, transferred to centrifuge tubes and diluted with DI water to 12.5 mL. Samples were filtered using Whatman® no. 1 filter paper and analyzed through ICP–OEM. Ground soybean (*Glycine max*) shoot was used as reference material and blanks were included in every batch.

3.2.10. Soil Nutrient Analysis

3.2.10.1 Silicon analysis

Silicon content was determined by 0.5 M acetic-acid extraction procedure followed by MBC (Korndorfer et al., 2001), whereas analysis of extractable nutrient content was based on Mehlich-3 procedure followed by ICP-OEM (Mehlich, 1984). For soil Si content analysis, 2 g of soil was weighed into a polyethylene centrifuge tube and added with 20 mL of 0.5 M acetic acid. The tubes were shaken using a reciprocal shaker (Eberbach; model number E6010.00) set at high speed for 1 hour. Soil suspension was filtered using Whatman® no. 1 filter paper. A 0.5 mL aliquot was transferred to a centrifuge tube for MBC analysis. Ten milliliters of DI water, 0.5 mL of 1:1 HCl:water solution, and 1 mL of 10% ammonium molybdate (adjusted to pH 7.5) were successively added to the samples. Samples were allowed to stand for 5 minutes before adding 1 mL of 20% tartaric acid. Samples were gently swirled for 10 seconds, allowed to sit for 2 minutes, added with 1 ml of ANSA and then with DI water to make 25 mL final volume. Absorbance reading was measured after 5 minutes at 630 nm using UV visible spectrophotometer (Hach DR 5000). Standard series at rates of 0, 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 ug mL^{-1} of Si, blanks, and reference samples were also measured.

3.2.10.2 Mehlich-3 analysis

The plant-essential nutrient contents in the soils were measured by weighing 2 g of soil in a 125 mL plastic bottle and adding 20 mL of Mehlich-3 solution. Samples were shaken for 5 minutes using a reciprocal shaker at high speed and filtered using Whatman® filter paper no. 42.

Clear filtrates were transferred to 10-mL plastic tubes and analyzed by ICP-OEM. Two blanks and two references were also included in the analysis.

3.2.11. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) Analysis

Leaf samples were collected at reproductive stage of the rice plants then six small sections of leaves were cut for microscopic characterization of Si deposition. Scanning Electron Microscopy and EDX microanalysis and mapping were used to determine Si deposition in the adaxial leaf surface of rice. Two replications for each treatment were analyzed. Under SEM, the magnification of samples' images was set to 1,500x and 2,000x, and system operation at voltage of 5.0 kV. Focus and brightness were also adjusted to obtain clear and good quality images. After generation of SEM images, EDX was set to scan samples. System operation was set at voltage 20.0 kV. Silicon peaks were proportionally quantified according to leaf carbon (C), oxygen (O), aluminum (Al), sulfur (S), chlorine (Cl), potassium (K), and calcium (Ca) contents. Similarly, scanning electron microscopy coupled to EDX microanalysis mapping was also conducted to determine Si content and deposition on leaf surface. Sample number of pixels was multiplied by a corresponding scale value and summed to give the overall Si content. Silicon content was associated with a green color in this study.

3.2.12. Statistical Analysis

Data were analyzed using a proc MIXED linear model (SAS Institute, Inc., Cary, NC). Proc MIXED allows modeling of random and mixed effect data, handling of unbalanced data and simplifying analysis related to repeated measures, data with heterogeneous variances and autocorrelated observations. The results were expressed as mean for each treatment group and the significance was evaluated at $p<0.05$ (F-test).

3.3. Results and Discussion

3.3.1. Effect of Soil Types, Silicon Addition, and Different Carriers Inoculated with SSB on rice agronomic variables

There was no significant interaction effect of soil types, Si addition, and carrier on straw and root dry weights, tiller number, number of panicles, and grain yield of rice except for the total aboveground biomass (*p*-value = 0.0534) in 2019 (Table 3.2). However, the soil type effect was significant on root dry weight (*p*-value = 0.0366), tiller number (*p*-value = 0.0267) and total aboveground biomass (*p*-value = 0.0023) in 2019. No data were gathered for straw, number of panicles, and grain yield in 2019. The plants were not able to produce grains due to the long day-length and low temperature in the greenhouse. The total aboveground biomass in 2019 was significantly higher in Commerce silt loam (640.37 g m^2) compared to Gigger silt loam (405.36 g m^{-2}) (Figure 3.1). The tiller number and root dry weight of rice on Commerce silt loam soil was significantly higher by 33% and 49%, respectively, relative to Gigger silt loam soil. In 2020, soil type significantly influenced straw (*p*-value=0.0001) and root dry weights (*p*-value = 0.0029), tiller number (*p*-value = <0.0001), and grain yield (*p*-value = 0.0160) of rice (Table 3.1).

Silicon addition and carrier had no significant effect on straw and root dry weights, tiller number, number of panicles, grain yield, and total aboveground biomass of rice measured for both years. Singh and Singh (2005) also did not find any substantial increase in plant growth under greenhouse conditions with Si fertilization. Plants treated with Si have improved growth and efficiency under environmental and biological stress, according to Epstein (2001). Sousa and Korndorfer (2010) noted that the environment is regulated in greenhouse and plants undergo minimal or no stress conditions, which may partially explain the lack of plant response across Si fertilization sources in this experiment.

Table 3.2. Results on analysis of variance for straw and root dry weights, tiller number, number of panicles, grain yield, and total aboveground biomass of rice.

Sources of Variation	Straw	RODW †	Tiller number	Number of panicles	Grain yield	Total Aboveground
2019						
Soil (S)	-	**0.0366**	**0.0267**	-	-	**0.0023**
Si Addition (Si)	-	0.5189	0.2684	-	-	0.3853
Carrier (C)	-	0.7465	0.8487	-	-	0.5542
S x Si	-	0.7358	0.4232	-	-	0.9001
S x C	-	0.8848	0.7074	-	-	0.7072
Si x C	-	0.2499	0.4064	-	-	0.6969
S x Si x C	-	0.8758	0.2807	-	-	**0.0534**
2020						
Soil (S)	**0.0001**	**0.0029**	**<0.0001**	0.4524	**0.0160**	0.2274
Si Addition (Si)	0.7228	0.9312	0.8398	0.8549	0.6290	0.8677
Carrier (C)	0.2854	0.5442	0.6487	0.4760	0.5782	0.2643
S x Si	0.7365	0.5826	0.4451	0.6615	0.4548	0.9978
S x C	0.2158	0.4679	0.7455	0.9819	0.5504	0.5729
Si x C	0.3257	0.2664	0.2295	0.4485	0.3986	0.4810
S x Si x C	0.5536	0.9096	0.3219	0.5312	0.1729	0.4912

†RODW – root oven dry weight

*Data on straw and grains Si content and uptake were not gathered in 2019.

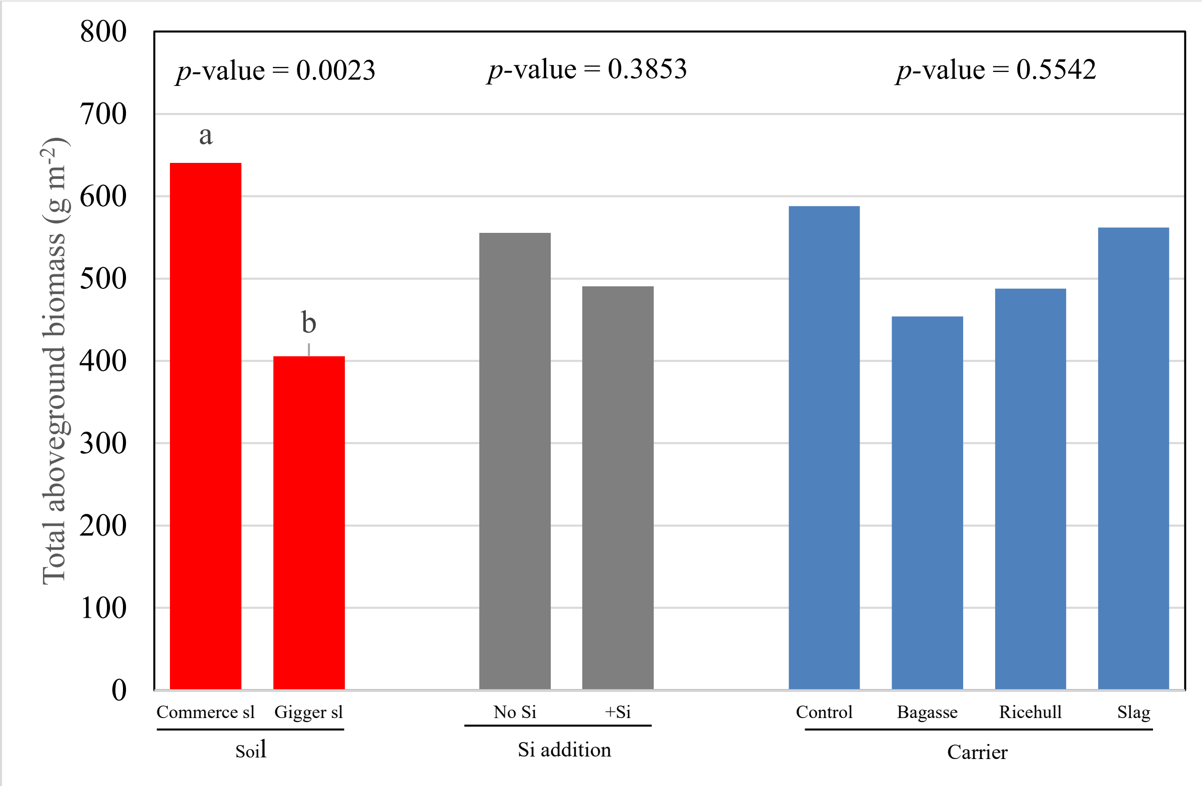


Figure 3.1. Effect of soil type, Si addition, and different carriers inoculated with SSB on the total aboveground biomass in 2019. Bars within each factor with different letters are significantly different at p<0.05.

The ANOVA showed that there was no significant interaction between soil type, Si addition and carrier on grain yield (Table 3.2). However, the mean rice grain yield was significantly different between soil type (*p*-value = 0.0160; Figure 3.2). The grain yield in Gigger silt loam was significantly higher (306.33 g m^{-2}) compared to Commerce silt loam (225.95 g m^{-2}) (Figure 3.2). As cited by Ahmad et al. (2013), the effects of Si on yield are linked to the deposition of the material under the leaf epidermis, resulting in a physical protection mechanism, decreasing lodging, raising the ability of photosynthesis, and reducing losses of transpiration. Research in India reported that while Si fertilization is not a common practice, several studies have demonstrated the beneficial function of Si application in improving rice yields (Peera et al., 2016).

In this study, Si addition did not significantly affect the grain yield of rice. However, application of Si in the form of wollastonite at 500 Kg ha^{-1} produced 8% higher grain yield at harvest than no Si addition. Silicon application may increase rice yield and mitigate abiotic stress, especially during conditions of drought (Cuong et al., 2017). Singh and Singh (2005) did not observe a substantial increase in plant growth under greenhouse conditions with Si fertilization. Similarly, SSB grown on different carriers did not significantly increase grain yield. However, rice inoculated with SSB in bagasse produced numerically the highest grain yield (287.77 g m^{-2}) among the carriers.

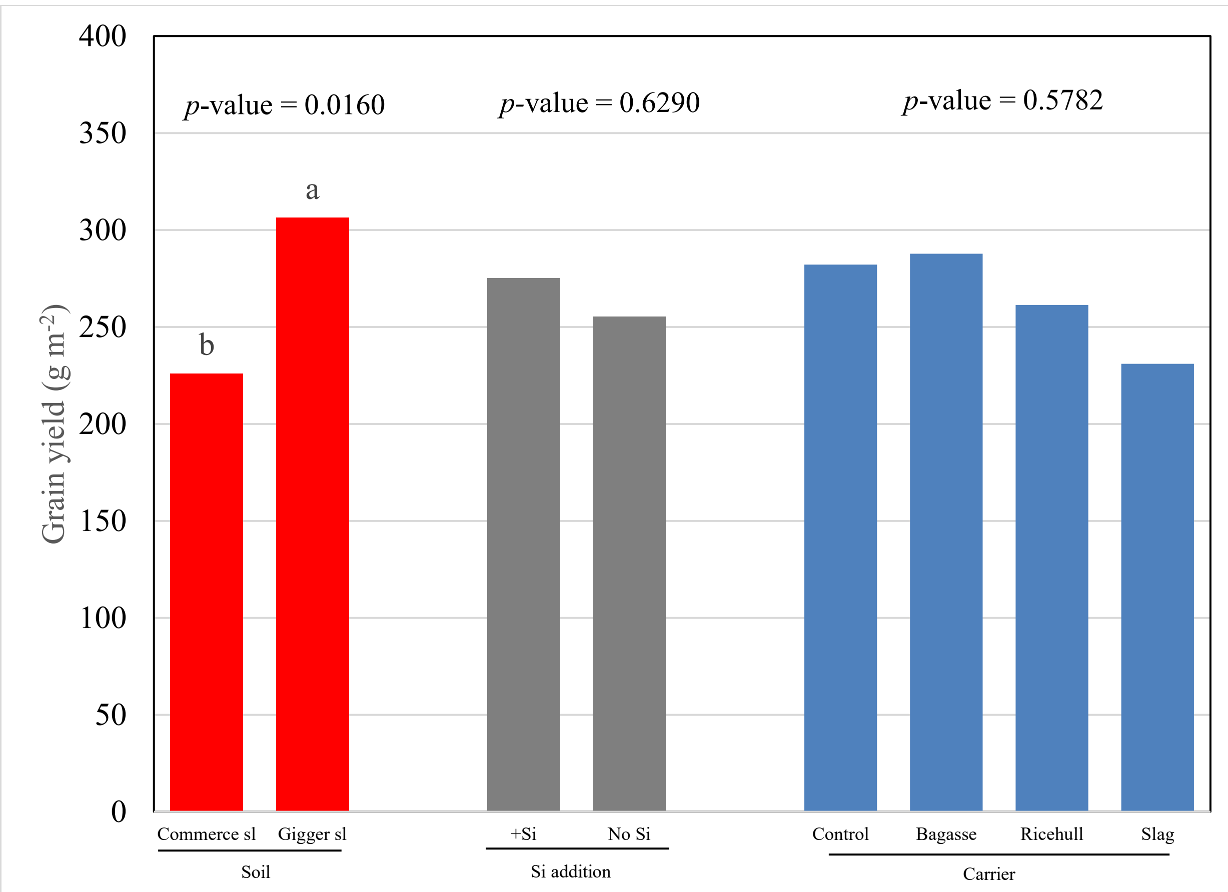


Figure 3.2. Effect of soil types, silicon addition, and different carriers inoculated with SSB on grain yield. Bars within each factor with different letters are significantly different at $p<0.05$.

3.3.2. Effect of Soil Types, Silicon Addition, and Different Carriers Inoculated with SSB on Rice Silicon Content and Uptake

The ANOVA showed that there was no significant interaction effect between soil types, Si addition and carrier on aboveground biomass Si and root Si content in 2019 (Table 3.3). However, the main effect of soil type was significantly observed in both aboveground biomass Si and root Si content (*p*-values = <0.0001; Figures 3.3-3.4). The aboveground biomass Si content in Commerce silt loam was significantly higher (2.7%) compared to Gigger silt loam (1%) (Figure 3.3). Similarly, root Si content in Commerce silt loam (0.74%) was significantly higher compared to Gigger silt loam (0.42%) (Figure 3.4).

There was no significant interaction effect between soil types, Si addition and carrier on Si content of straw, roots, and grains in 2020 (Table 3.3). However, the main effect of soil type was significantly observed in straw Si content. The straw Si content in Commerce silt loam (1.54%) was significantly higher compared to Gigger silt loam (1.11%) (*p*-value = <0.0001; Figure 3.5). The addition of Si (wollastonite) also significantly improved straw Si content of rice by 20% relative to the no Si addition treatment (*p*-value = <0.0001; Figure 3.5). Similarly, root Si content in Commerce silt loam was significantly higher (0.74%) compared to Gigger silt loam (0.42%) (Figure 3.6). Soil type also significantly influenced the grain Si content of rice (*p*-value = 0.0002; Figure 3.7).

Appendix Table 3.1 and Appendix Table 3.2 summarized the results on analysis of variance for shoot and root essential nutrient contents at harvest in 2019. Majority of the nutrients was significantly affected by the soil type. However, Si addition significantly improved shoot Mg and Mn contents and root Ni content. Significant interaction between Si addition and carrier was also observed in shoot Ni content.

Table 3.3. Results on analysis of variance for rice Si content and uptake.

Sampling	Sources of Variation	%Si Straw	%Si Roots	%Si Grains	%Si Aboveground biomass	Straw Si uptake	Root Si uptake	Grains Si uptake	Aboveground biomass Si uptake	Total Si uptake
2019										
	Soil (S)	-	**<0.0001**	-	**<0.0001**	-	**0.0043**	-	**<0.0001**	**<0.0001**
At Harvest	Si Addition (Si)	-	0.2204	-	0.4789	-	0.5610	-	0.5516	0.5137
	Carrier (C)	-	0.4460	-	0.0913	-	0.7609	-	0.1833	0.1995
	S x Si	-	0.8499	-	0.7007	-	0.7358	-	0.6974	0.6761
	S x C	-	0.7377	-	0.3202	-	0.8056	-	0.7557	0.8162
	Si x C	-	0.2669	-	0.5664	-	0.5042	-	0.2473	0.4029
	S x Si x C	-	0.2525	-	0.5926	-	0.4879	-	0.0696	0.1154
2020										
	Soil (S)	**0.0135**	-	-	**0.0135**	0.4012	-	-	0.4012	0.4012
Mid-Season	Si Addition (Si)	**0.0035**	-	-	**0.0035**	0.2409	-	-	0.2409	0.2409
	Carrier (C)	0.2283	-	-	0.2283	0.7951	-	-	0.7951	0.7951
	S x Si	0.0919	-	-	0.0919	**0.0462**	-	-	**0.0462**	**0.0462**
	S x C	0.0802	-	-	0.0802	0.4633	-	-	0.4633	0.4633
	Si x C	0.6858	-	-	0.6858	0.9094	-	-	0.9094	0.9094
	S x Si x C	0.0605	-	-	0.0605	0.1640	-	-	0.1640	0.1640
At Harvest	Soil (S)	**<0.0001**	**<0.0001**	**0.0002**	**<0.0001**	**0.0393**	0.3851	0.8078	0.2306	0.2386
	Si Addition (Si)	**<0.0001**	0.0638	0.3408	**0.0046**	**0.0486**	0.3851	0.0709	**0.0449**	0.0558
	Carrier (C)	0.1141	0.9007	0.8639	0.8581	0.5819	0.6566	0.7343	0.7536	0.7473
	S x Si	0.5614	0.5796	0.3718	0.3203	0.3142	0.3851	0.8213	0.5768	0.5488
	S x C	0.9288	0.3782	0.2953	0.4005	0.7787	0.5311	0.8945	0.8232	0.8098
	Si x C	0.8058	0.4651	0.6905	0.7848	0.4113	0.1517	0.2038	0.4223	0.3942
	S x Si x C	0.5688	0.9610	0.1681	0.6652	0.4746	0.8457	0.4938	0.5702	0.5814

* Data on straw and grains Si content and uptake were not gathered in 2019.

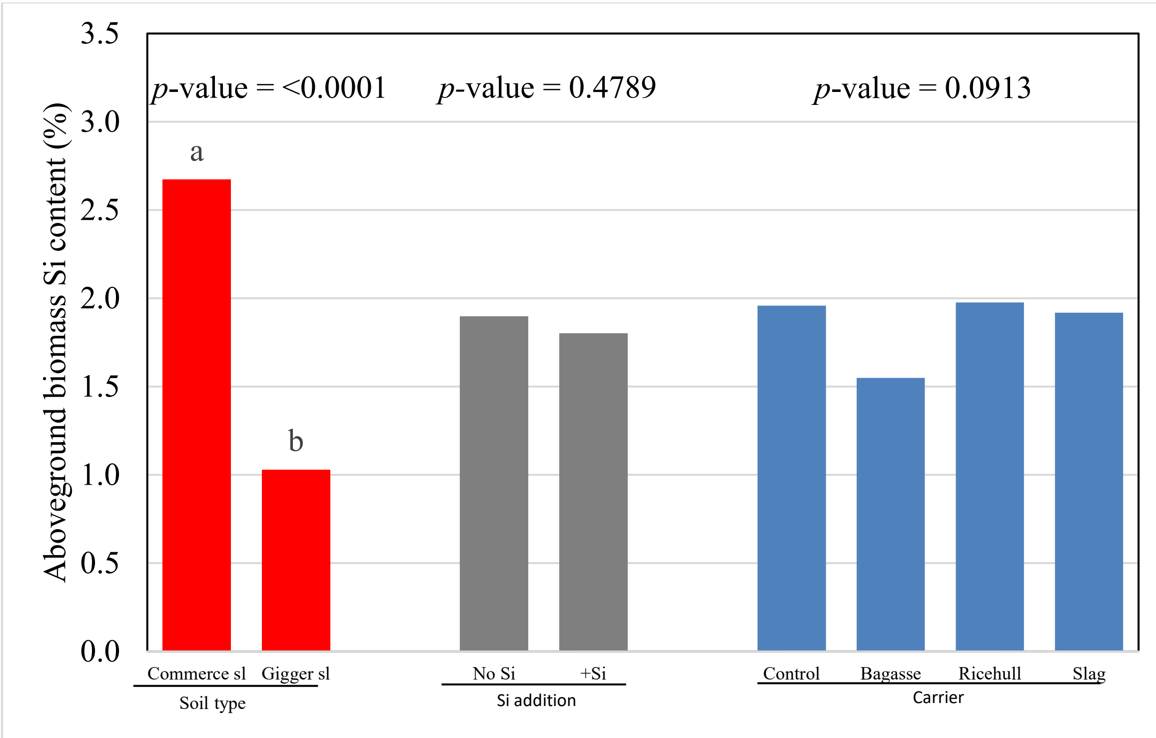


Figure 3.3. Effect of soil types, silicon addition, and different carriers inoculated with SSB on the aboveground biomass Si content in 2019. Bars within each factor with different letters are significantly different at $p<0.05$.

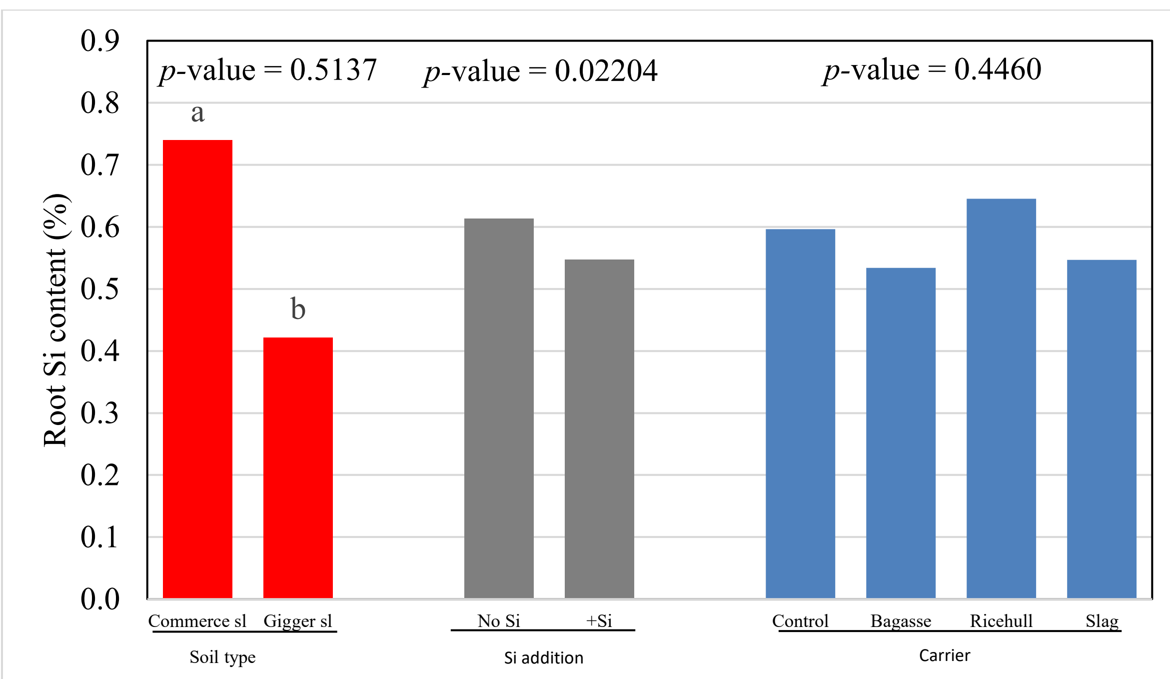


Figure 3.4. Effect of soil types, silicon addition, and different carriers inoculated with SSB on the root Si content in 2019. Bars within each factor with different letters are significantly different at $p<0.05$.

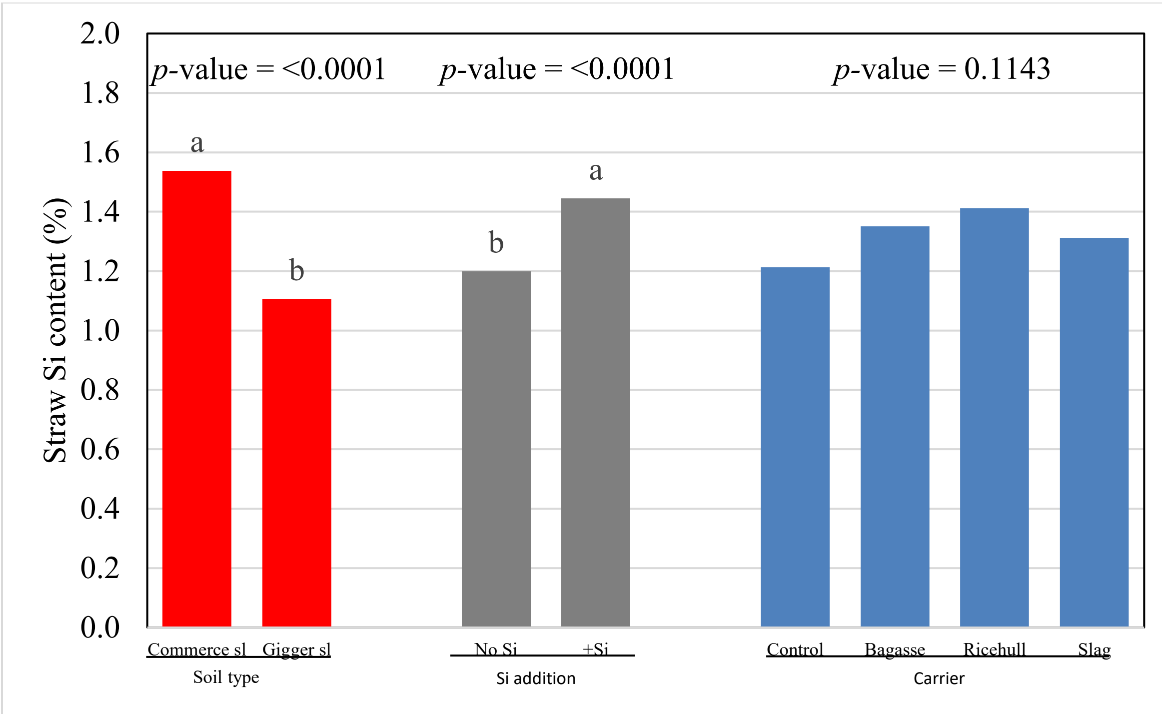


Figure 3.5. Effect of soil types, silicon addition, and different carriers inoculated with SSB on the straw Si content in 2020. Bars within each factor with different letters are significantly different at $p<0.05$.

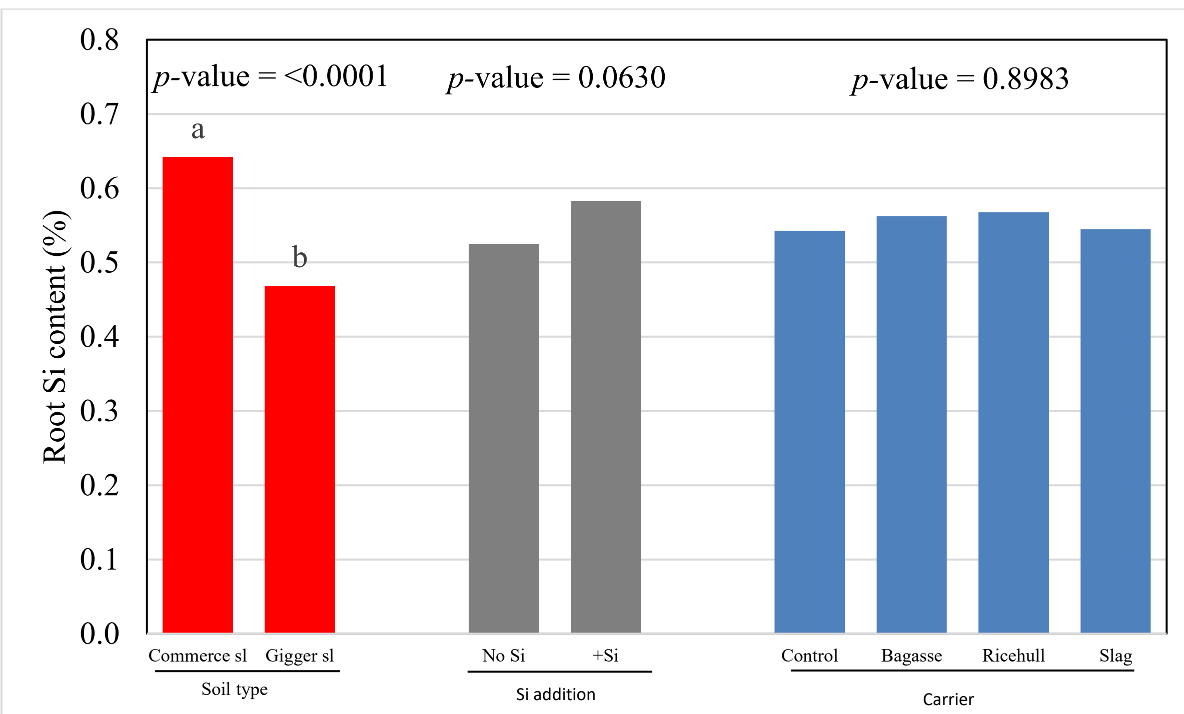


Figure 3.6. Effect of soil types, silicon addition, and different carriers inoculated with SSB on the root Si content in 2020. Bars within each factor with different letters are significantly different at $p<0.05$.

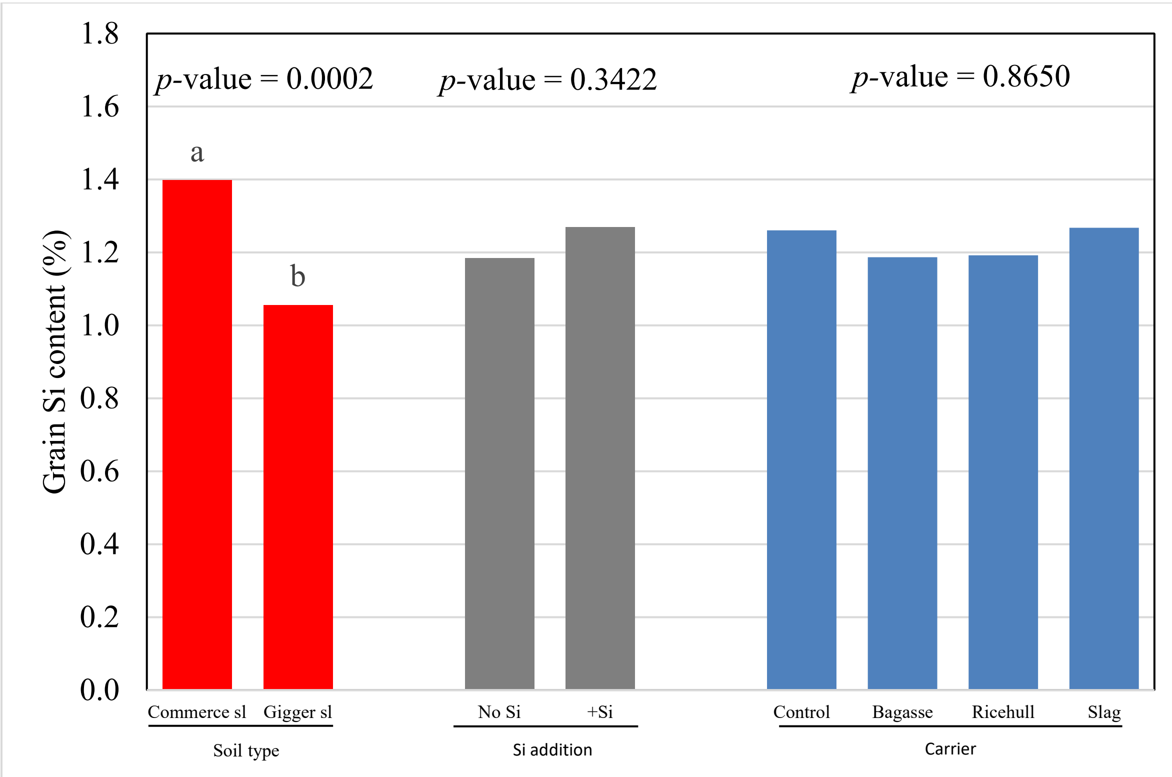


Figure 3.7. Effect of soil types, silicon addition, and different carriers inoculated with SSB on the grain Si content. Bars within each factor with different letters are significantly different at $p<0.05$.

In terms of Si uptake, the ANOVA showed no significant interaction between soil types, Si addition and carrier in 2019 (Table 3.3). However, the main effect of soil type was significantly observed in total Si uptake (*p*-value = <0.0001; Figure 3.8). The total Si uptake in Commerce silt loam was significantly higher (0.62%) compared to Gigger silt loam (0.21%) (Figure 3.8).

Similarly, no significant interaction between soil types, Si addition and carrier was observed on straw and grain Si uptake and total Si uptake in 2020 (Table 3.3). Mean straw Si uptake between soil types was significantly different with rice planted on Gigger silt loam having higher (0.39%) Si uptake compared to Commerce silt loam (0.31%) (*p*-value = 0.0393; Figure

3.9). The addition of Si (wollastonite) also significantly improved straw Si uptake of rice by 22% relative to the no Si addition treatment (*p*-value = 0.0486; Figure 3.9). The maximum Si uptake was observed in straw (149.5 kg ha^{-1}) followed by plant at panicle initiation (76.28 kg ha^{-1}) and grain initiation (39.42 kg ha^{-1}) in the analysis by Peera et al. (2016). The Si uptake ranged from 21.4 to 62.3 kg ha^{-1} for grain.

Grain Si uptake did not differ with soil type, Si addition and different carriers; however, Si addition tended to increase grain Si uptake by 24% (*p*-value = 0.0709; Table 3.3 and Figure 3.10). The Si addition tended to improve the total Si uptake by 23% relative to the no Si addition with *p*-value = 0.0558 (Table 3.3; Figure 3.11). The inoculated SSB in bagasse seemed to enhance total Si uptake (0.63%) by rice however, the *p*-value > 0.05. The study by Cuong et al. (2017) clearly demonstrated the significant impact of Si addition to rice Si uptake. Silicon accumulation in rice straw and total biomass for all Si doses was substantially different from those of the control. Compared to control, nearly 57% more Si was accumulated in rice straw under the recommended dose of fertilizer (RDF) + 400 kg ha^{-2} SiO_2 treatment. The application of Si fertilizer resulted in higher Si uptake in rice biomass (grain + straw) over the control. The Si deficiency or sufficiency in soil is primarily determined by the rate by which Si is replenished in the soil solution and rate of Si uptake during plant development. A rice production system that entirely relies on the H_4SiO_4 released from its stubbles needs to be replenished every six years as available Si is expected to be depleted after five years of continuous cultivation. An increased supply of Si is stated to be beneficial to monocotyledons in general and species of *Poaceae* such as rice in particular. Silicon can be actively and passively absorbed by rice, but low temperature or metabolic inhibitors can substantially limit absorption (Cuong et al., 2017).

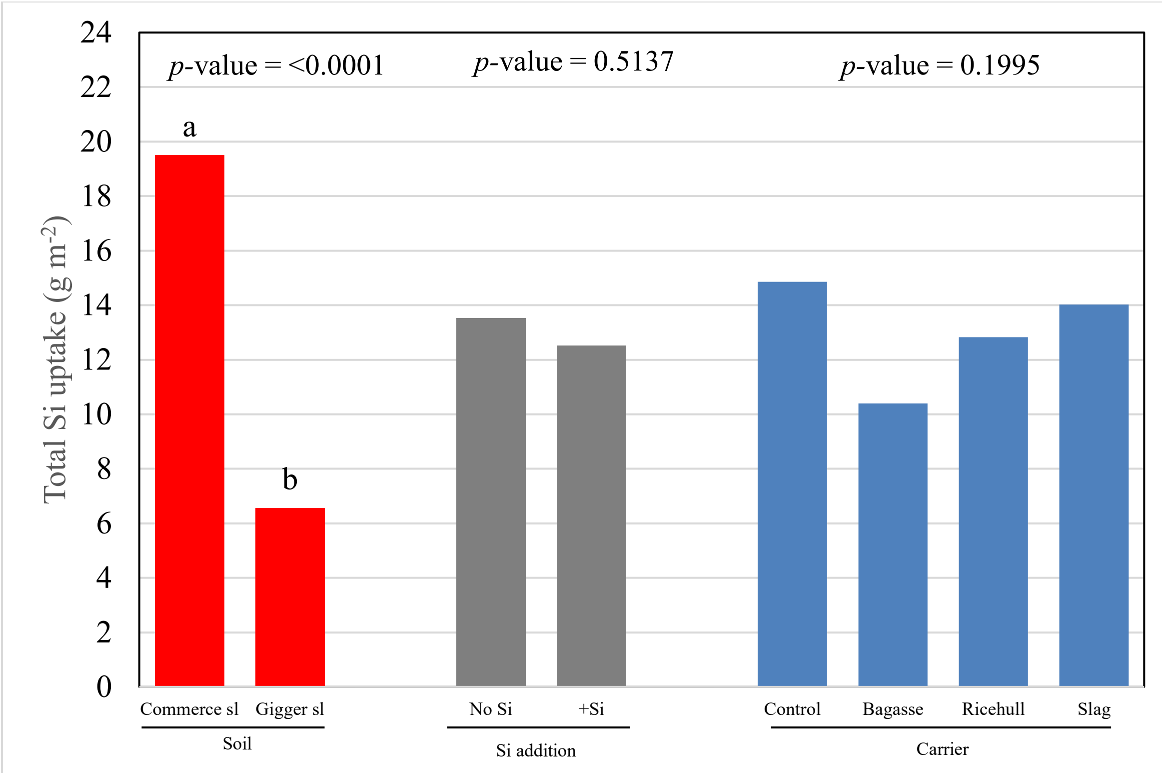


Figure 3.8. Effect of soil types, silicon addition, and different carriers inoculated with SSB on the total Si uptake in 2019. Bars within each factor with different letters are significantly different at $p<0.05$.

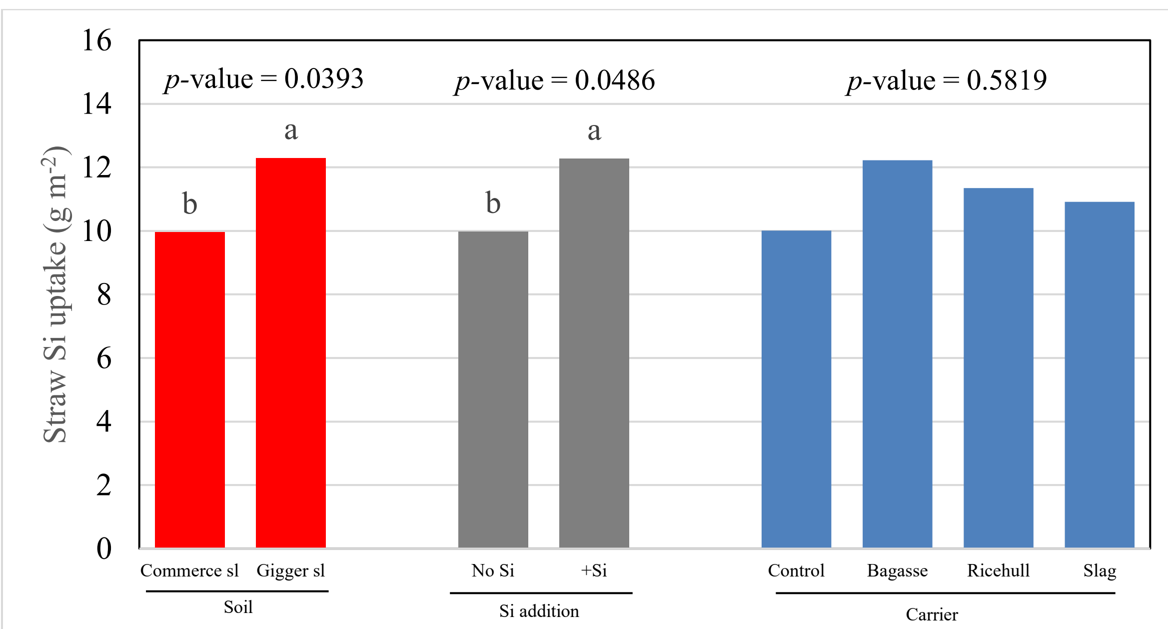


Figure 3.9. Effect of soil types, silicon addition, and different carriers inoculated with SSB on the straw Si uptake in 2020. Bars within each factor with different letters are significantly different at $p<0.05$.

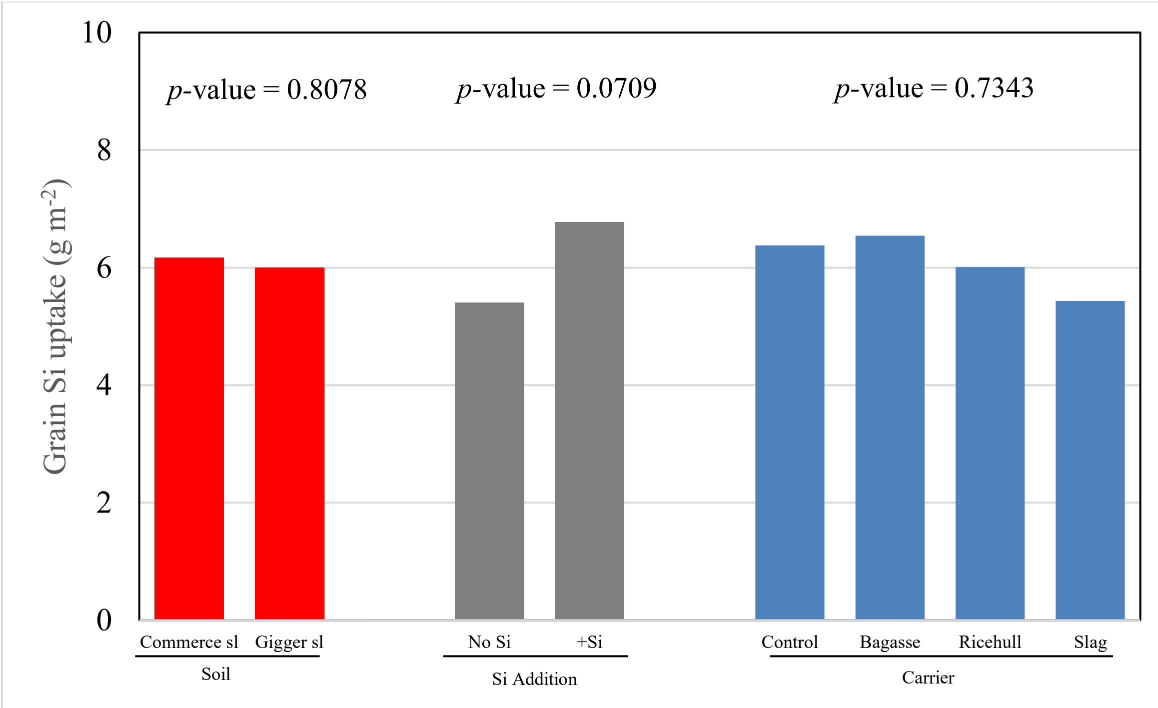


Figure 3.10. Effect of soil types, silicon addition, and different carriers inoculated with SSB on grain Si uptake in 2020.

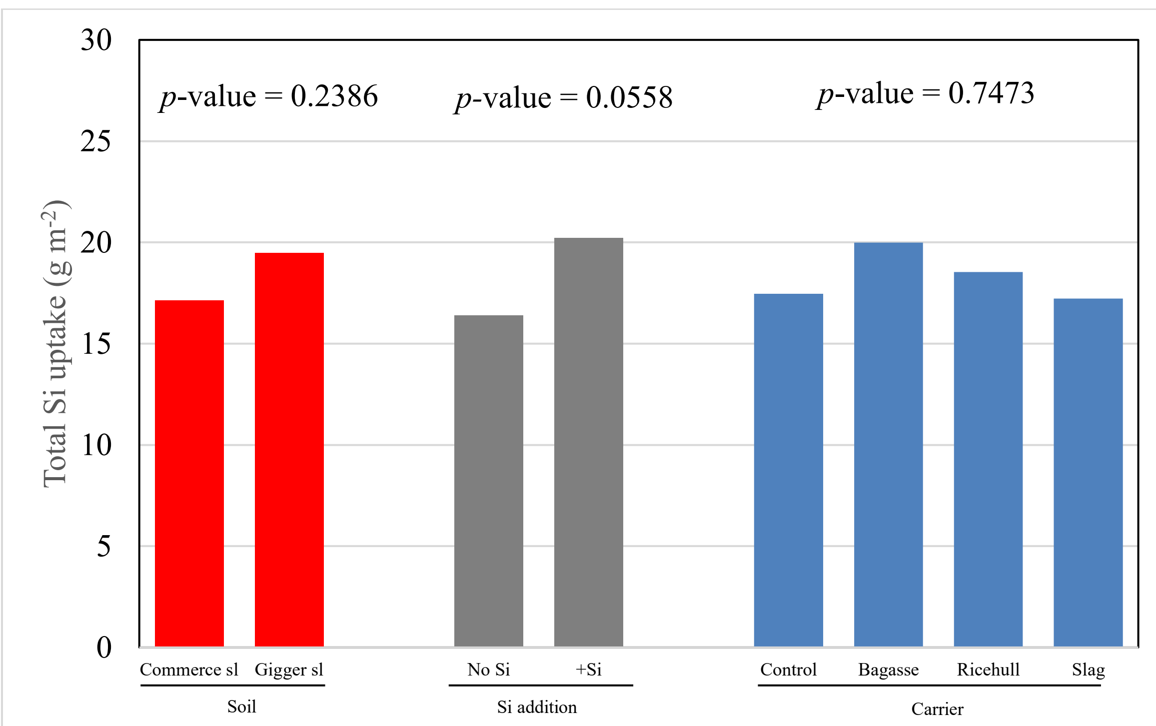


Figure 3.11. Effect of soil types, silicon addition, and different carriers inoculated with SSB on the total Si uptake in 2020.

3.3.3. Effect of Soil Types, Silicon Addition, and Different Carriers Inoculated with SSB on Soil pH and Nutrient Content

Table 3.4 summarizes the effects of soil types, Si addition, and different carriers inoculated with SSB on the soil pH, Si, and essential nutrient content. Significant differences on these parameters were consistently observed between soil types whereas Si addition showed a significant impact but only on soil Si and Ca. The effect of Si addition on soil Si was not the same for both soils (S x Si interaction). In addition, there was a 3-way interaction effect on soil P (Table 3.4).

The soil pH value of Commerce silt loam (pH = 7.41) was significantly higher than the Gigger silt loam (pH = 6.87) (*p*-value = <0.0001; Figure 3.12). A significant interaction effect between soil type and Si addition was observed on soil Si concentration. Here, the addition of Si on Gigger silt loam resulted in significant increase on soil Si from 43 to 66 mg kg^{-1} (*p*-value = <0.0023; Figure 3.13) whereas the soil Si of Commerce silt loam was unaffected by Si addition. The concentration of available Si in the soil (initial or fertilized) decreases as soil acidity increases because lower soil pH prevents the dissolution of Si in the soil (Korndorfer et al., 2003).

There was a significant 3-way interaction effect of soil types, Si application, and different carriers inoculated with SSB on soil P (*p*-value = <0.0023; Table 3.4 and Figure 3.14). Soil P levels were compared across all treatment combinations. The highest P was 294 mg kg^{-1} in Commerce silt loam with Si addition and received SSB-inoculated bagasse. It is very evident that the low levels of P were recorded in Gigger silt loam regardless of Si addition and carrier treatments. It has been suggested in the study of Schaller et al. (2019) that in terrestrial systems Si fertilization can increase the P content of plants by increasing P availability in the soil.

However, a study cited by Tubana and Heckman (2015) mentioned that the interaction between phosphate and H_4SiO_4 in the soil environment is antagonistic. With increasing H_4SiO_4 concentrations, the amount of phosphate ion released into the soil solution increases. It is also explained by the strong competition for specific sorption sites which is more likely a long-term effect of the silicic acid. Gibbsite, which has decreased its affinity for phosphate ions when silicified into kaolinite, is a good example. The amorphous silicic acid (from silicate ions) had a lower negative surface charge than that of the phosphate ion. Thus, the amorphous silicic acid is preferentially adsorbed over the phosphate ion when these two ions are present in the soil solution.

Table 3.4. Results on analysis of variance for soil pH, Si, and essential nutrient content after harvest.

Sources of Variation	pH	Soil Si	P	K	Ca	Mg	S	Cu	Zn
Soil (S)	**<0.0001**	**<0.0001**	**<0.0001**	**0.0061**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Si Addition (Si)	0.5027	**0.0183**	0.3255	0.8685	**<0.0001**	0.5665	0.0992	0.0713	0.4767
Carrier (C)	0.5476	0.2822	0.1193	0.9158	0.3354	0.6981	0.3169	0.5351	0.3213
S x Si	0.9724	**0.0023**	0.9517	0.7814	0.9413	0.4284	0.8679	0.5003	0.2569
S x C	0.0971	0.8953	**0.0490**	0.5515	0.3346	0.5855	0.9883	0.6795	0.5489
Si x C	0.9221	0.3434	0.5550	0.1896	0.1770	0.1411	0.2651	0.6162	0.2130
S x Si x C	0.7334	0.8728	**0.0352**	0.2737	0.2186	0.4946	0.9465	0.6623	0.6292

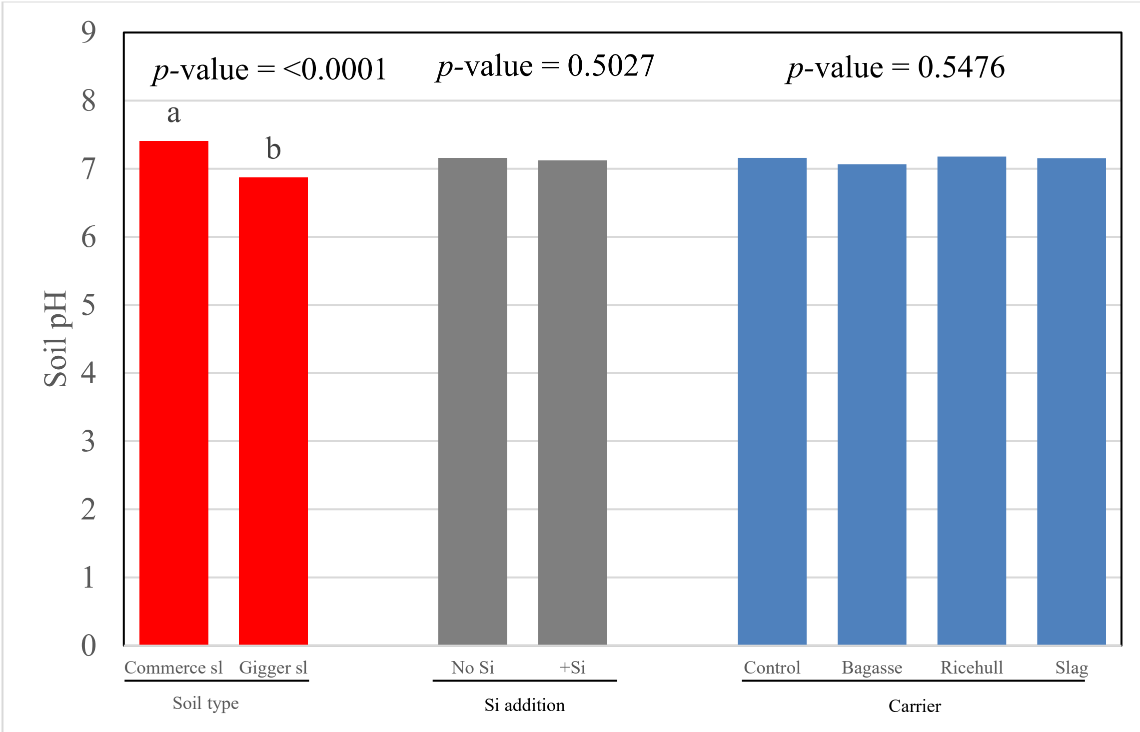


Figure 3.12. Effect of soil types, Si addition, and different carriers inoculated with SSB on the soil pH. Bars within each factor with different letters are significantly different at p<0.05.

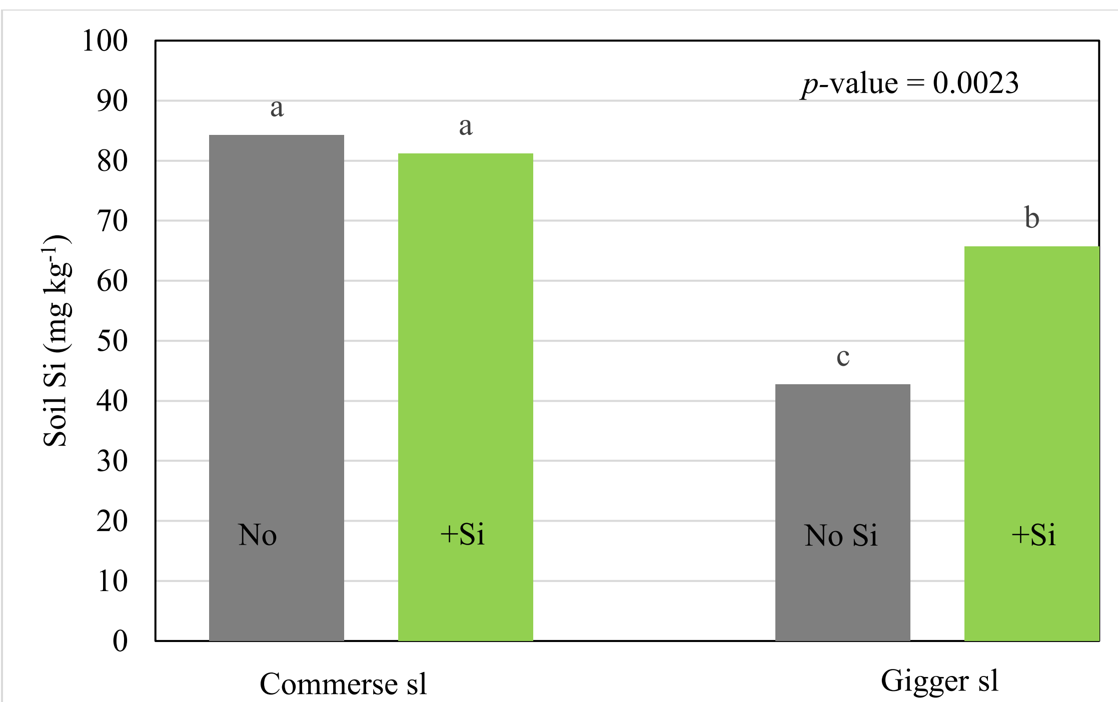


Figure 3.13. Effect of soil types and Si addition on soil Si content. Bars with different letters are significantly different at p<0.05.

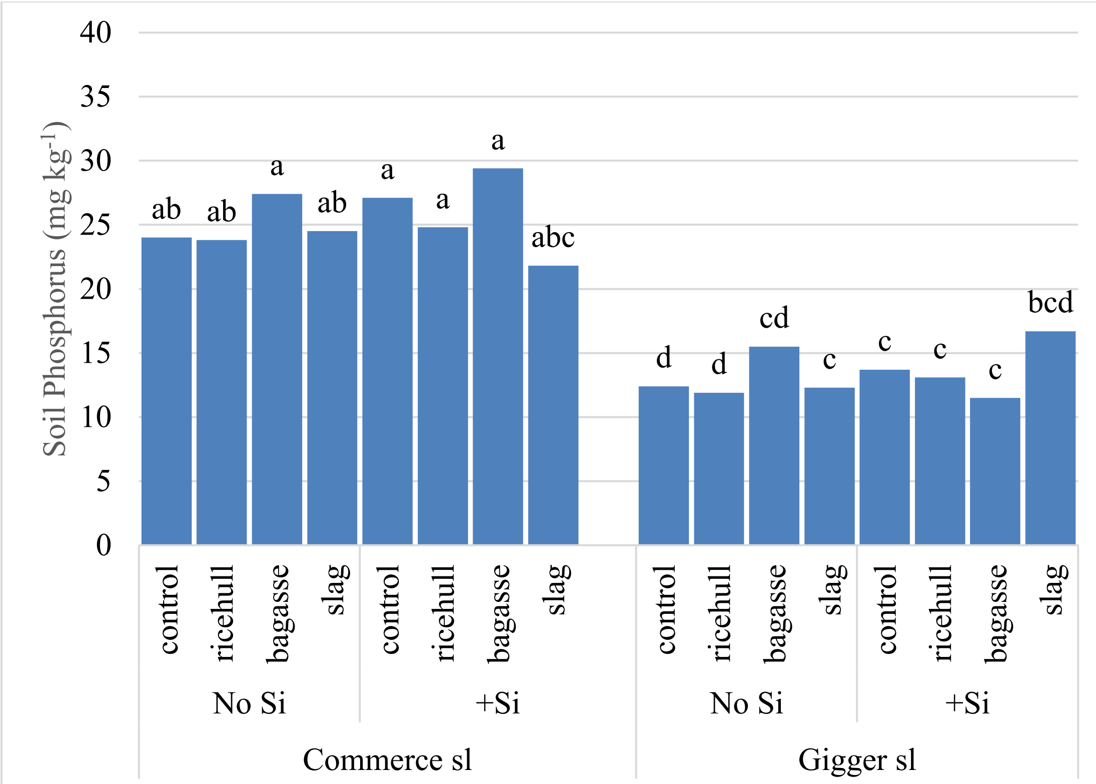


Figure 3.14. Effect of soil types, Si addition, and different carriers inoculated with SSB on the soil phosphorus. Bars with different letters are significantly different at $p<0.05$.

3.3.4. Effect of SSB Application on Plant Si Content Using Scanning Electron Microscope-Energy Dispersive X-ray (SEM-EDX)

Figure 3.15 shows the rice plant as affected by Si addition. Silicon makes the rice plant more erect and not stunted as compared to the no Si treatment. The addition of Si (wollastonite) also significantly improved straw Si uptake of rice by 22% relative to the no Si addition treatment as shown from the previous discussion (p-value = 0.0486; Figure 3.9). Silicon is a major inorganic higher-plant constituent that is accumulated in the form of amorphous silica gel ($SiO_2 \cdot nH_2O$) in plants. Grasses can either stunt or suppress growth in the absence of Si, so Si has been considered an important element for normal plant growth and development. There have been numerous reports on its biological purpose, but in terms of its direct role in the metabolism of higher plants that accumulate it in significant amounts, the function of Si is still unclear. Silicon is deposited primarily in the epidermis of rice plant tissues, vascular bundles plus bundle sheath, and sclerenchyma in leaves (Kim et al., 2002). In rice plants, silica is deposited in the form of silica bodies produced by epidermal cells, silica cells and bulliform cells (Agarie et al., 1996).

Scanning electron microscopy followed by energy dispersive X-ray analysis is useful for the purpose of locating silica bodies on the leaf surface of rice plants. Figure 3.16 shows the cross sections of leaves (red arrow highlighting the presence of silica bodies or phytolith) of rice grown on soil treated with SSB using SEM-EDX. The SEM image show silica bodies were mostly concentrated on the epidermal cell walls of rice leaf. Kim et al. (2002) reported that electron microscopy and in situ X-ray microanalysis provided evidence that Si was deposited within sub-epidermal tissues of Si-treated rice leaves in epidermal cell walls, middle lamellas, and intercellular spaces. In the epidermal cell wall structure, the most significant variations were observed between Si-treated rice plants and control plants. These results were also consistent with the findings of Yoshida et al. (1962). In the analysis by Agarie et al. (1996), as seen in the cross-section of Si-treated rice plants, leaf silica cells occurred in the epidermal layer above and below the vascular bundles. Silicon deposition was not, however, limited to electron-dense epidermal cell walls and was also observed as polymorphic aggregates in middle lamella and intercellular spaces (Kim et al., 2002). Silicon is also believed to be associated with components of the cell wall, such as polysaccharides and proteins (Carpita, 1996). Silicon is likely to be integrated into cell walls as Si-aromatic ring contacts in rice leaves between lignin and carbohydrate (Inaga and Okasaka, 1995).

Figure 3.15. Rice plants without **(a)** and with Si addition **(b)**.

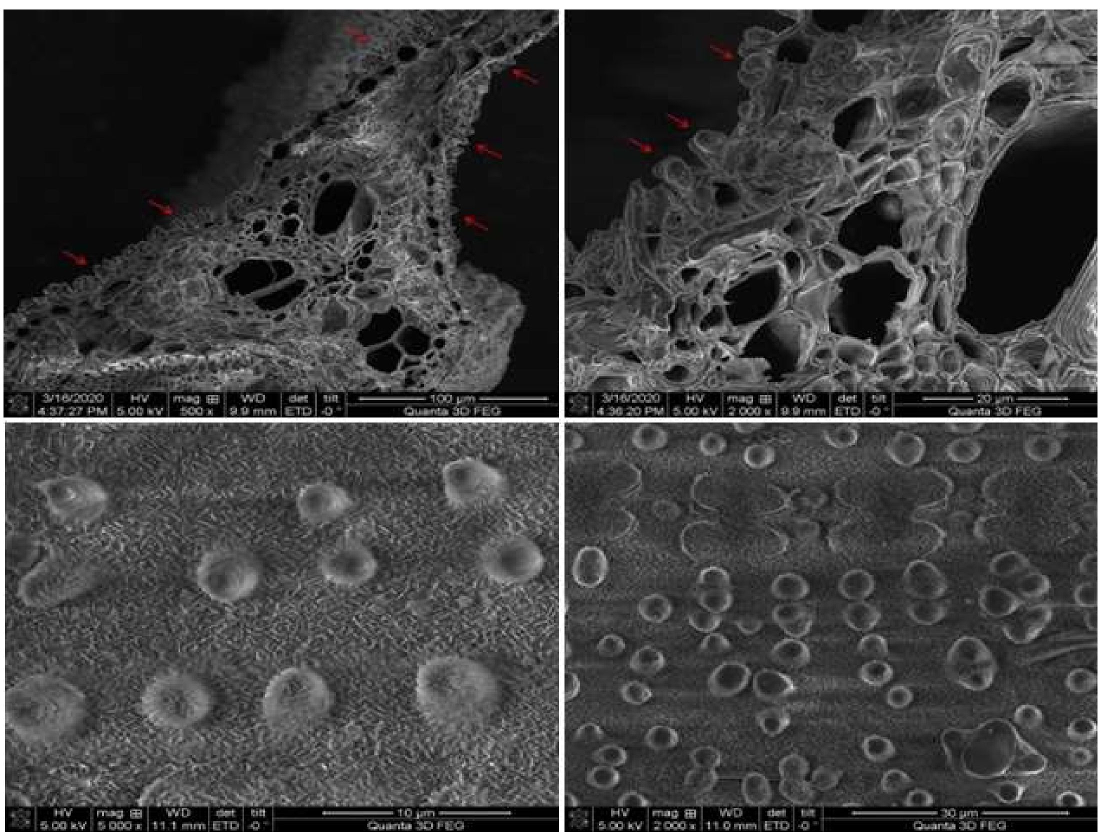


Figure 3.16. Cross sections of rice leaves (red arrow highlighting the presence of silica bodies or phytolith) treated with SSB **(a)** and silica deposition in the adaxial leaf surface of rice using SEM-EDX at 5,000x **(b)** and 2,000x **(c)** magnification.

The deposition of silica in rice leaves was evaluated via SEM-EDX and Si mapping. Figures 3.17 and 3.18 show SEM-EDX Si map of adaxial leaf surface of control, with Wollastonite, SSB-inoculated rice hull, SSB-inoculated bagasse, and SSB-inoculated slag rice plants planted on Commerce silt loam soil and Gigger silt loam, respectively. The results from SEM-EDX analysis showed the differences on Si distribution between Si-treated and untreated plants.

In Commerce silt loam, the Si mapping visually showed greater distribution of silica bodies on the adaxial leaf surface of rice with Si (wollastonite) applications and different carriers inoculated with SSB in comparison to the uninoculated (control) rice plants. The SEM-EDX analysis showed that plants treated with SSB-inoculated rice hull had the highest number of silica bodies on rice leaf with an equivalent %Si content of 55% relative to %C, %O, and %K. On the other hand, the lowest number of silica bodies was distributed on the leaf surface of uninoculated rice plants. Plants treated with wollastonite, SSB in bagasse, and SSB in slag had 48%, 35%, and 40% Si, respectively.

In Gigger silt loam, Si mapping visually showed greater distribution of silica bodies on the adaxial leaf surface of rice applied with wollastonite (48% Si) followed by the uninoculated rice (46%). On the other hand, plants treated with SSB in rice hull, SSB in bagasse, and SSB in slag had 40%, 26%, and 37% Si, respectively.

Several trichomes, wart-like protuberances, Si papilla, and dumbbell-shaped silica bodies were observed on the leaf surface of rice treated with wollastonite and SSB (Figures 3.17 and 3.18). Kim et al. (2002) observed similar types of silica bodies distributed on the surface of rice leaves. Point analysis of Si X-ray on the surface of Si-treated plants revealed that Si was present in both trichomes and wart-like protuberances. On the other hand, epidermal regions without wart-like protuberances and stomatal guard cells accumulated relatively small amount of Si compared with trichomes and wart-like protuberances. High X-ray counts of Si coincided generally with the areas protruded on the leaf surface.

In the present study, dumbbell-shaped silica bodies were observed on rice treated with SSB-inoculated rice hull and SSB-inoculated bagasse. As the leaves matured, the silica cells changed shape gradually and this change reflected the flexibility of the silica cell wall at earlier stages and the rigid sandwich-like structure at later stages of development (Zhang et al., 2013). There were two processes involved in silica cell development in the leaf epidermis of rice. First, the silica cell wall is lignified and silicified, and its structure becomes dumbbell-shaped; second, unknown mechanisms assemble the needle-like biosilica that initiate the structure from the inner wall of the silica cell, expand it in a specific direction, and then gradually stack needles in the lumens of dumbbell cells, filling up the silica cell lumens until the leaf is fully exposed.

The detailed mechanisms regulating the biomineralization of Si in higher plants, however, are still unknown and additional experiments are required. Silicon deposition in cell walls and silica cells of leaves formed inside dumbbells could play an important role in defending against biotic challenges (Zhang et al., 2013). The leaves displayed a dispersed Si profile in the absence of added Si. The leaves displayed a dispersed Si distribution profile in the absence of added Si (Ranganathan et al., 2006). In Si-treated plants, the Si leaf content was substantially higher, and the silica cell size was larger than in non-Si-treated plants (Cai et al., 2008).

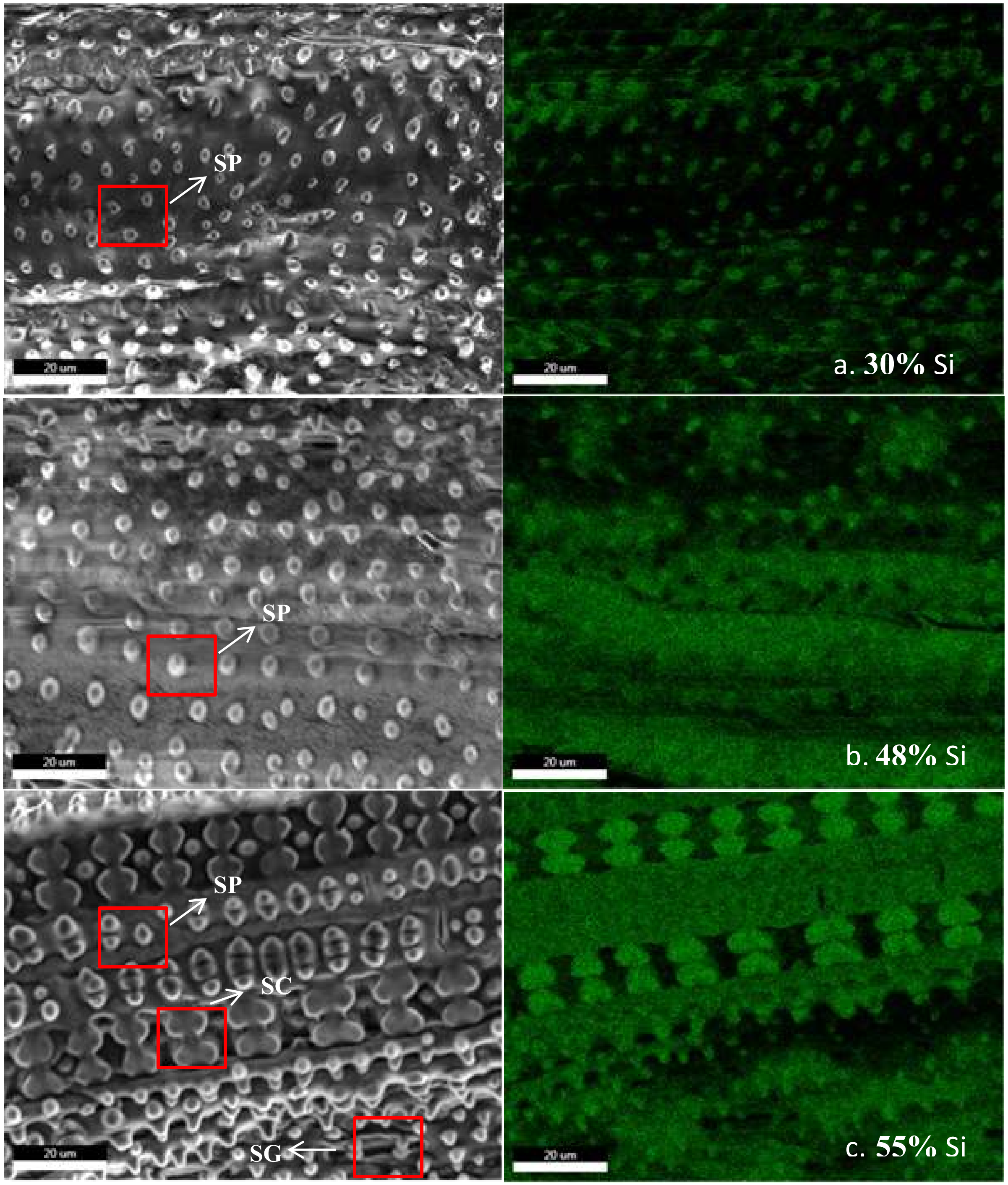
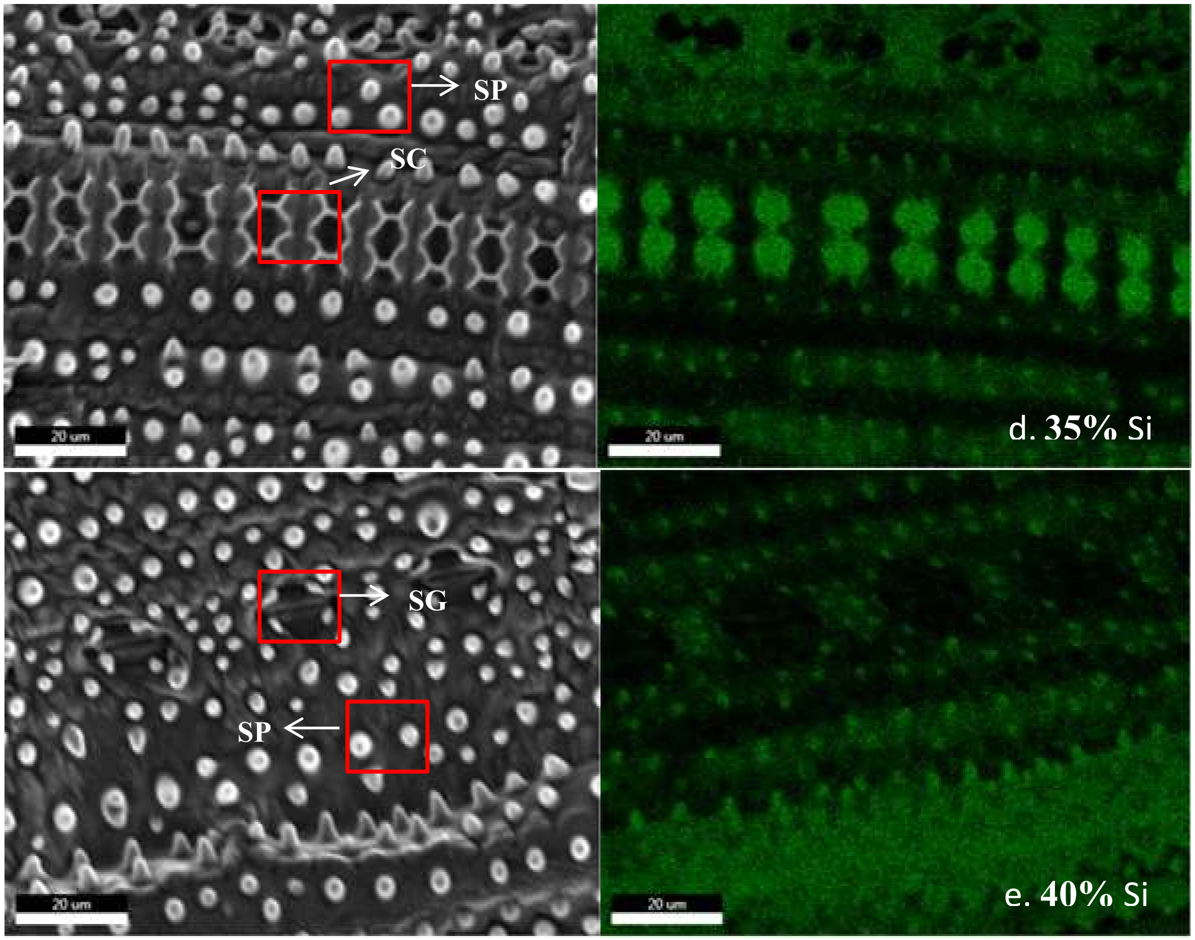


Figure 3.17. Scanning electron microscope-energy dispersive X-ray (SEM-EDX) silicon map of adaxial leaf surface of uninoculated **(or control – a)**, with wollastonite **(b)**, SSB-inoculated rice hull **(c)**, SSB-inoculated bagasse **(d),** and SSB-inoculated slag **(e)** rice plants planted in Commerce silt loam soil**.** Values are %Si relative to %C, %O, and %K. SC, silica cell in dumbbell-shaped; SP, silicon papilla; SG, stomatal guard cell.



(fig. 3.17 Cont'd).

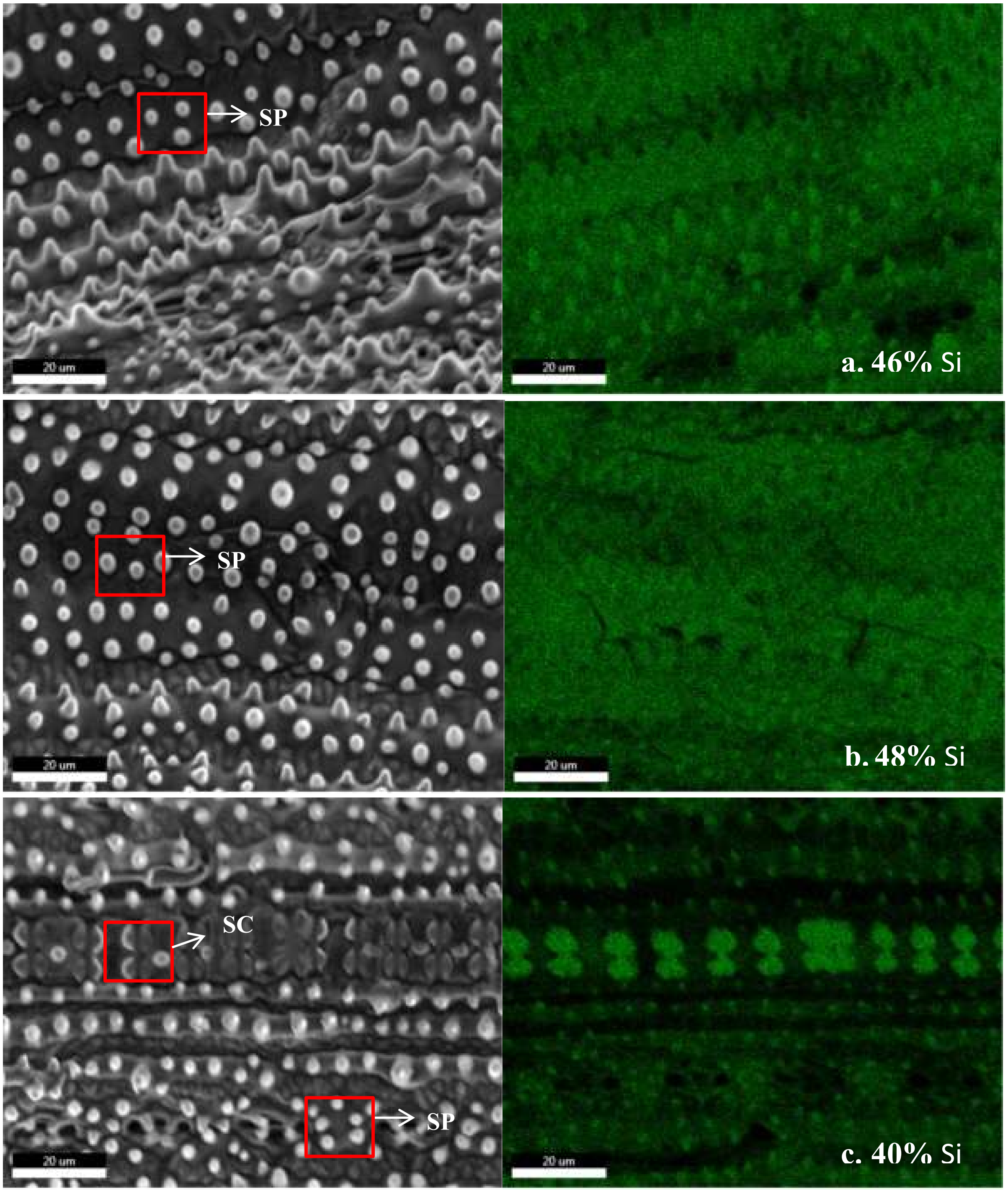
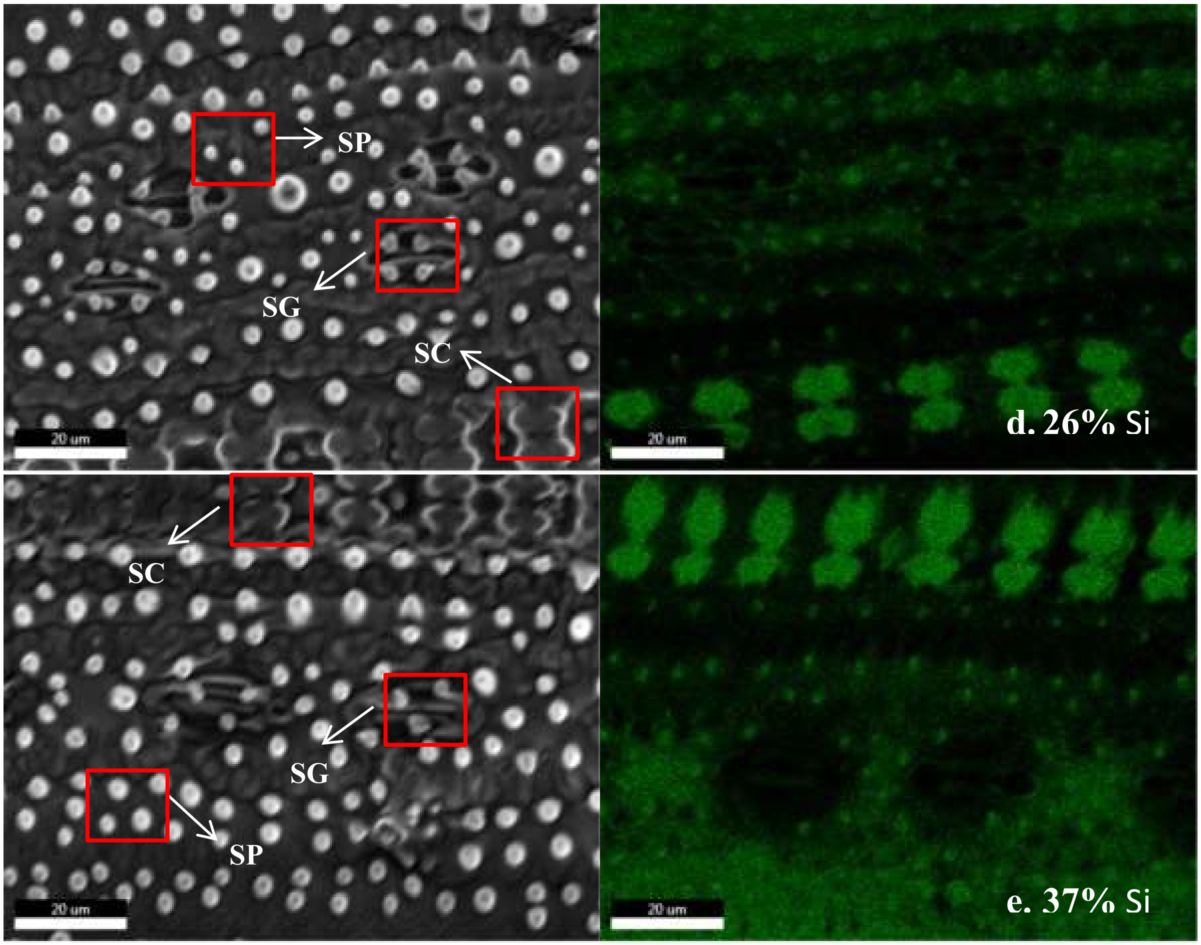


Figure 3.18. Scanning electron microscope-energy dispersive X-ray (SEM-EDX) silicon map of adaxial leaf surface of uninoculated **(or control – a)**, with Wollastonite **(b)**, SSB-inoculated rice hull **(c)**, SSB-inoculated bagasse **(d),** and SSB-inoculated slag **(e)** rice plants planted in Gigger silt loam soil**.** Values are %Si relative to %C, %O, and %K. SC, silica cell in dumbbell-shaped; SP, silicon papilla; SG, stomatal guard cell.



(fig. 3.18 Cont'd).

3.4. Conclusions

Greenhouse experiment was conducted to evaluate the effectiveness of previously identified multi-SSB grown on different carriers in enhancing the growth, Si uptake, and yield of rice. The differences in agronomic variables and Si nutrition of rice were evident and consistent between the soil types. While Si addition did not result in significant grain yield increase, there was a significant improvement observed on rice Si uptake. The SBB inoculant performed essentially similar and showed no contribution to rice biomass and grain yield. The lack of yield response to Si addition was partly explained by the high initial soil Si availability and if not completely absence, minimal occurrence of growth limiting factors during the growing period. The semi-quantitative evaluation of silica bodies distribution on leaf surface of rice (treated with wollastonite and SSB using different carriers) via SEM-EDX differed between soil types. Greater distribution of silica bodies was observed in rice planted in Commerce silt loam.

The outcomes of this study suggest that SSB inoculation is a “ready” approach in improving Si availability to crops but still the benefits of this potential technology were not fully realized. Further evaluation on the effectiveness of this SSB is needed. For future research, the following factors could be improved: concentration of SSB and method and time of application. To better demonstrate, we may need to use soils with very low Si content and that the test plants will be subjected to stressful condition or just simply setup the experiment in the field. Field assessment is needed to provide more information on the effect of biotic and abiotic stresses on soil types, silicon addition, and SSB grown on different carriers on rice productivity.

Chapter 4: Development of Practical Approach in Scaling Up Silicate-Solubilizing Bacteria Use in Crop Production

4.1. Introduction

Plant growth-promoting rhizobacteria (PGPR) represents a wide variety of soil bacteria which, when grown in association with a host plant, result in stimulation of plant growth. Biofertilizer refers to the use of soil microorganisms to increase the availability and uptake of mineral nutrients for plants through several mechanisms. They have the ability to convert minerals from unavailable form to available form through various biological processes. Some bacteria are capable of fixing the atmospheric nitrogen (N), and solubilize phosphorus (P) and silicon (Si). These microorganisms are being broadly dispersed in different environments such as soil, water, and sediments (Vessey, 2003).

Several studies stated the beneficial effects of PGPR on plant growth and yields, due to the increase in fixed N content in soil (Mrkovac et al., 1996) and to the microbial secretion of plant stimulating hormones, like gibberellins, auxins, and cytokinins and solubilization of phosphates as cited by Abd El-Fattah et al. (2013). Microorganisms play a vital role in the dissolution of soil minerals by different mechanisms especially in ion cycling and soil fertility (Uroz et al., 2007; Calvaruso et al., 2006; Ehrlich, 1996). Soil beneficial microorganisms such as N-fixers and phosphate-solubilizing bacteria (PSB) are effective plant growth-promoters. Another group of bacteria, silicate-solubilizing bacteria (SSB), is involved in the conversion of silicates into soluble silica (Rangaraj et al., 2013). These groups of bacteria control the chemical and biological properties of soil through absorption of soluble monosilicic acid. Hence, these groups of beneficial microorganisms could be a potential bio-inoculant for plants.

Silicon is an important micronutrient for the safe and competitive growth of all Asian cereals, including rice (*Oryza sativa*) (Brunings et al., 2009). The role of Si in plant health and growth was investigated in Si accumulating crops and appeared to be significantly effective (Jinab et al., 2008). Research shows that adequate Si uptake can increase the tolerance to both abiotic and biotic stress of agronomic crops, especially rice, (Ma and Takahashi, 2002). While Si is not considered to be an essential element for higher plants, many plant species have been shown to benefit from enhanced Si supply, especially tropical graminaceous plants such as rice, which is a hyper-Si accumulator (Liang et al., 2007; Lee et al., 2019).

Wollastonite is one of the Si sources used in rice production. Babu et al. (2016) reported that the highest grain yield of rice was observed on wollastonite-treated soils. Grain yield was increased by 16.5% with the application of 680 kg Si ha^{-1} with wollastonite as source on Sharkey clay soil. Silicon, considering its abundance in the earth's crust, is mainly found in insoluble forms that are not readily available for plant uptake. Until solubilized by the weathering action of rocks or biological activity of plant roots and microorganisms, it remains in insoluble form (Naureen et al., 2015).

Silicate-solubilizing bacteria play an important role on increasing plant-available Si in the soil, subsequently improving Si uptake by plant and enhancing its defense mechanisms to both biotic and abiotic stress. The research work on Si has been more focused on a soil fertilization and standardization of soil Si testing with very few studies pursuing the contribution of microorganisms on Si nutrition of plant. In addition, environmental concerns and rising cost of chemical fertilizers are some major concerns in crop production. Hence, alternative technologies like utilizing beneficial microorganisms like SSB should be implemented. This would offer the industry a practical, innovative, and ecologically-smart crop care solution, not to mention its huge potential as a commercial product.

Variable inoculant quality is a common problem in the tropics and subtropics where inocula are often subjected to unpredictable handling and storage during distribution and use. The physicochemical and biological characteristics of carrier materials influence its suitability under adverse conditions particularly its survival rate (Kremer and Peterson, 1983). Identifying suitable carriers for the inoculum is necessary to obtain a good quality bio-inoculant. The general objective of the present study was to develop a feasible approach of incorporating SSB in the rice production system.

Specifically, the objectives of the study were the following:

a. Determine the survival of SSB using different carriers derived from slag, bagasse, rice hull, and soil.
b. Document the colonization potential of silicate-solubilizing bacteria (labeled with GFP) in rice plants.

4.2. Materials and Methods

4.2.1. Isolate Used

This bacterium was isolated from Gigger-Gilbert complex soil, fine-silty, mixed, active, thermic Typic Fragiudalfs in Winnsboro, Louisiana, USA (32.1418, -91.6862). This isolate can solubilize silicate and produce other plant growth-promoting compounds such as 1-aminocyclopropane carboxylic acid (ACC) deaminase, indole-3-acetic acid (IAA), and phosphatase (Please see Chapter 2 for details). The probable bacterium identity is *Pseudomonas sp.* with 99% of maximum identity based on 16S rDNA analysis. The bacterium was maintained on Luria-Bertani (LB) broth and agar medium. These were in dehydrated forms that were prepared only when needed. All media was sterilized for 20 min at 121°C before use.

4.2.2 Survival of SSB on Different Inoculant Carriers

4.2.2.1. Inoculant carrier

Slag, fresh bagasse, fresh rice hull, slag + soil, burned bagasse + soil, burned rice hull + soil, fresh bagasse + soil, and fresh rice hull + soil carriers were evaluated for their effectiveness to sustain the optimum population of SSB (Table 4.1). Commerce silt loam soil collected from St. Gabriel was used as component of the carrier. The carriers were sterilized in an autoclave for 1 hr at 121°C for 3 consecutive days. Different methods were used for the sterilization of carrier materials to obtain the most suitable one without any effect on their quality. Steam sterilization by autoclaving is the most commonly used and has the superiority among all employed methods due to low cost and its ability to allow absolutely pure culture of inocula to be prepared (Strijdom and Deschodt, 1976).

The C:N ratio and chemical characteristics of different carrier materials are shown in Table 4.2. The C:N ratio was determined by dry combustion method using LECO® CN628 analyzer. For essential nutrient contents, samples were digested with concentrated nitric acid (HNO_3) and 30% H_2O_2 at 305°F, and analyzed using inductively coupled plasma (ICP) –Optical Emission Spectroscopy (OEM).

4.2.2.2. Inoculation of the sterilized carrier

Two grams of each sterilized carrier (slag, fresh bagasse, fresh rice hull, slag + soil, burned bagasse + soil, burned rice hull + soil, fresh bagasse + soil, and fresh rice hull + soil) were used to make the inoculant. Actively growing SSB was inoculated in 100 mL LB medium and grown for 24 hours (Table 4.1). Broth culture was inoculated into the 2 g individual sterilized carrier which brought the carrier material moisture to approximately maximum water holding capacity. The inoculated carriers were mixed thoroughly and were incubated at room temperature (28-30ºC).

Table 4.1. Description of the different carrier materials and the amount of inoculum used in this study.

Treatment	Treatment Code	Description	Moisture content (%)	Inoculum added (ml100 ml^{-1} broth)
1	Slag	100% slag	16.00	4.12
2	Fresh bagasse	100% fresh bagasse	63.00	1.05
3	Fresh rice hull	100% fresh rice hull	66.00	1.00
4	Slag + soil	50% slag + 50% soil	15.00	4.40
5	Burned bagasse + soil	50% burned bagasse + 50% soil	52.00	1.27
6	Burned rice hull + soil	50% burned rice hull + 50% soil	32.00	2.06
7	Fresh bagasse + soil	50% fresh bagasse + 50% soil	40.00	1.65
8	Fresh rice hull + soil	50% fresh rice hull + 50% soil	28.00	2.36

4.2.2.3. Determination of SSB population

Cell population was counted periodically up to 180 days of incubation using the spread plate method. This method involves the dispersion of an aliquot of carrier suspension in an agar medium. The necessary degree of dispersion is achieved by making successive dilutions of a given carrier. Each viable microorganism present in the soil suspension develops into a visible colony (Black, 1965). Number of viable cells in the original population was determined by counting the number of colonies that developed after incubation. Plating was done on duplicate tryptic soy agar plates.

4.2.2.4. Colony forming units

The colonies were counted after about 1-2 days of incubation. In counting for the colony-forming unit (CFU), the formula that was used was:

$$CFU = \frac{no.of\ colonies \times dilution\ factor}{oven\ dry\ weight}$$

The CFU was transformed to logarithmic value and was plotted in a graph.

Table 4.2. Chemical characteristics of the carriers used in this study.

	B	Ca	C	Cu	Fe	Mg	Mn	Mo	N	P	K	S	Zn	C:N
Slag	-	23.00	-	-	14,000	7.00	16,000	-	-	-	-	0.50	-	-
Fresh bagasse	3.05	0.09	45.33	16.98	1,907	0.08	56	ndl	0.33	0.04	0.26	0.03	26	137
Burned bagasse	10.80	0.91	27.47	28.50	16637	0.48	453	2.39	0.77	0.26	1.51	0.14	147	36
Fresh rice hull	6.93	0.06	39.71	3.73	64.5	0.18	171	ndl	0.52	0.37	0.54	0.06	30	78
Burned rice hull	7.65	0.07	41.45	11.17	78.3	0.28	243	1.92	1.12	0.56	0.76	0.05	52	37

* N, P, K, Ca, Mg, S, and C were expressed in %; B, Cu, Fe, Mn, Mo, and Zn were expressed in mg kg^{-1}.

*ndl – non-detectable level

*Estimate composition of slag material (White, 2015).

4.2.3. Survival of SSB in Rice Seedlings

4.2.3.1. Construction of GFP-tagged isolates

WinnsB-6 isolate was selected for labeling with green fluorescence protein (GFP) gene by triparental mating. Green fluorescence protein, a small-sized protein obtained from the *Aequorea victoria* jellyfish (Tsien, 1998; Prasher et al., 1992), has now widely been used for colonization studies of PGPRs in rice and other crops (Hao and Chen, 2017; Liu et al., 2006; Zhu et al., 2002). A complementary DNA for the *Aequorea victoria* GFP produces a fluorescent product when expressed in prokaryotic (*Escherichia coli*) cells. In this study, donor bacteria strain *E. coli* with GFP plasmid construct pBB2rpoDGFP1 (Barphagha and Ham, unpublished) (kanamycin-resistant (Kmr) at 50 µg L^{-1}), recipient bacteria (WinnsB-6, nitrofurantoin-resistant (Ntr) at 50 µg L^{-1}), and the helper strain *E. coli* HB101 (prK2013Tn7) were grown overnight in LB broth with suitable antibiotics at 37°C. All bacteria were mixed in 1:1:1 ratio (volume/volume) with 500 µL each in a microcentrifuge tube, and 1.0 mL for each of the strains was taken as a control. The bacteria cultures grown in LB broth were centrifuged for 1 min. After discarding supernatant, the pellet was resuspended in 50 µL LB broth. The bacterial suspension was spotted on LB agar plate and incubated overnight at 37°C. All the bacterial cells were harvested and resuspended in 1 mL LB broth and plated on LB agar plates supplemented with Km and Nt. The plates were incubated at 37°C for 24 – 48 h to screen for successful transconjugants. Potential candidates were purified and further confirmed by observing green fluorescence under a fluorescence microscope (Leica DM6B Microscope).

4.2.3.2. Colonization and visualization of SSB in rice plants

Rice seeds treated with GFP-tagged strain (WinnsB-6-GFP) were allowed to grow for 2 weeks in falcon tubes with half strength Yoshida's nutrient solution in a hydroponic system

(Yoshida et al., 1976). The initial population of the GFP-tagged bacteria was determined by measuring the optical density using a UV-1600PC spectrophotometer.

The presence of GFP-tagged strains in roots was confirmed under a confocal laser scanning microscope (CLSM) (Confocal Microscope Leica SP8). Roots from 2-week-old seedlings were stained with propidium iodide (10 μg mL^{-1}) for 10 min. The stained samples were placed on a microscopic glass slide with 0.6 % agarose solution (m:v), covered with a glass side and observed under CLSM to observe for bacterial fluorescence. Green fluorescence protein-tag-free plants were used as controls.

4.3. Results and Discussion

4.3.1. Survival of SSB on Different Inoculant Carriers

A high-grade carrier should have high water retention and it should be inexpensive, nontoxic for the strain or environment and easy to sterilize (Swelim et al., 2010). Sugarcane (*Saccharum officinarum*) bagasse and perlite were tested as carriers for *Bradyrhizobium japonicum strain* CB1809 (Khavazi et al., 2007). According to Madigan et al. (2000), bacteria equip themselves in order to adapt to new environment, which in the case of this study are the following carriers: slag, fresh bagasse, fresh rice hull, slag + soil, burned bagasse + soil, burned rice hull + soil, fresh bagasse + soil, and fresh rice hull + soil (Figure 4.1). Cell population was counted periodically up to 180 days of incubation using the spread plate method. Figure 4.2 shows the actual SSB population on different carriers in 10^{-2} and 10^{-3} dilutions.

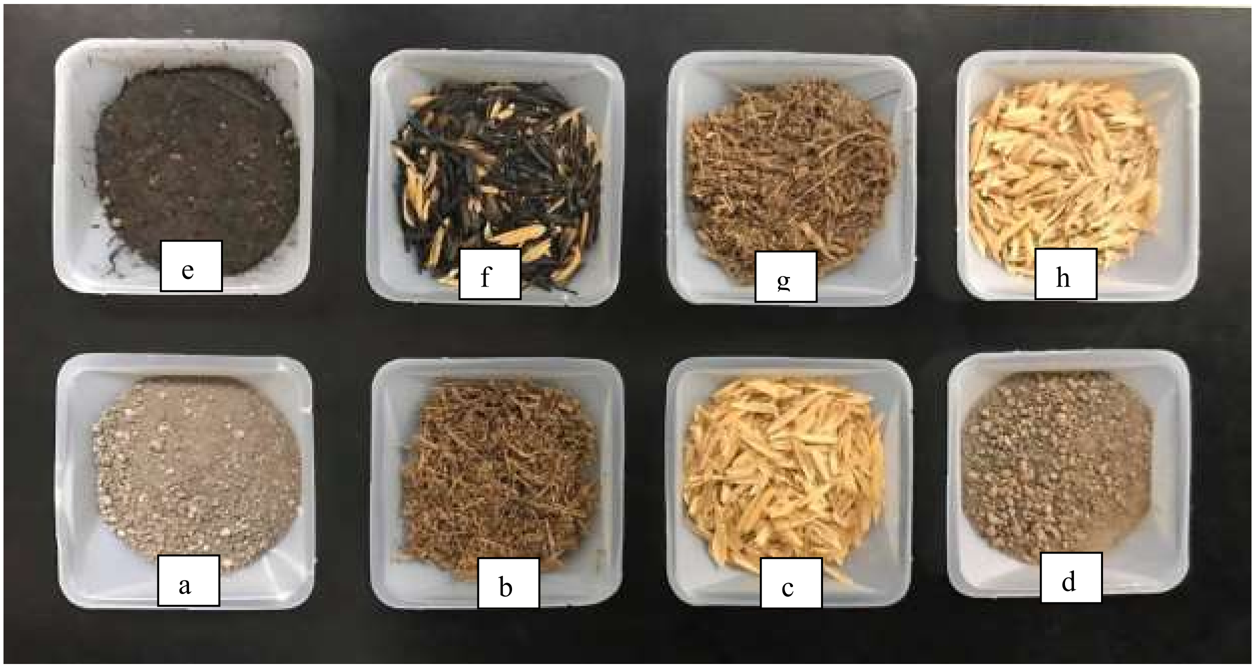


Figure 4.1. Different inoculant carriers: (a) slag, (b) fresh bagasse, (c) fresh rice hull, (d) slag + soil, (e) burned bagasse + soil, (f) burned rice hull + soil, (g) fresh bagasse + soil, and (h) fresh rice hull + soil.

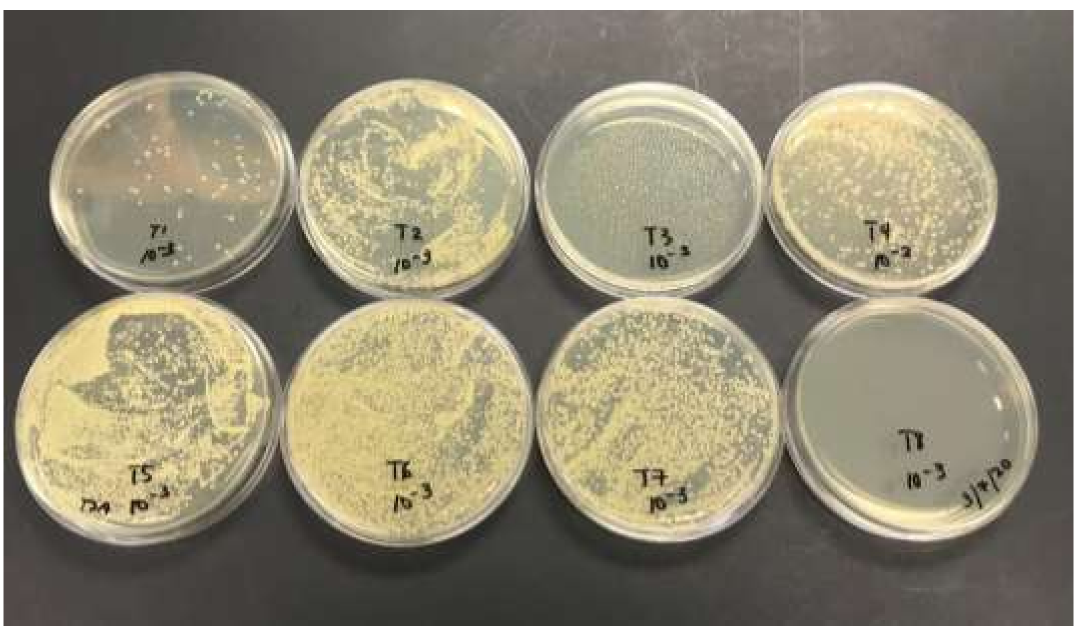


Figure 4.2. Silicate-solubilizing bacteria (SSB) population on different carriers in 10^{-2} and 10^{-3} dilutions using spread plate method. See Table 4.1 for treatment designation.

Figure 4.3 shows the population of SSB in slag, fresh bagasse, and fresh rice hull carriers. The initial population of SSB in the slag carrier of 3.0 x 10^5 cfu g^{-1} decreased to 9.1 x 10^3 cfu g^{-1} 5 days after inoculation (DAI). However, the number of cells continuously increased to 1.3 x 10^5 cfu g^{-1} at 20 DAI. It can be inferred that the bacterial growth is undergoing an exponential phase. This phase is when the bacteria continue to replicate and thus manifested by a growing population. A study by Belanger and Hatfull (1999) suggests that the exponential phase of bacteria plays a significant role in controlling the downstream availability of carbon and energy for the survival of the organism. Another possible reason for higher level of the bacterial population is their ability to produce growth hormone IAA which increases the exudation from the cells. The highest population of 4.1 x 10^5 cfu g^{-1} was obtained at 105 DAI with a final population of 2.0 x 10^3 cfu g^{-1} at 180 DAI; only 0.7% of the initial population remained viable in the slag carrier.

On the other hand, no bacteria were found in the fresh bagasse and fresh rice hull carriers 5 DAI. The highest populations of 1.1 x 10^7 cfu g^{-1} (50 DAI) and 3.4 x 10^3 cfu g^{-1} (15 DAI) were obtained in fresh bagasse and fresh rice hull, respectively.

Figure 4.4 shows the survival of SSB on slag + soil, fresh bagasse + soil, and fresh rice hull + soil carriers at 180 days after inoculation. The initial population of SSB in fresh bagasse + soil of 2.68 x 10^5 cfu g^{-1} increased to 4.5 x 10^5 cfu g^{-1} 5 DAI. Similarly, population of SSB in fresh rice hull + soil increased from 3.0 x 10^5 cfu g^{-1} to 3.8 x 10^5 cfu g^{-1} at 5 DAI. However, there was a drastic decrease in SSB population at 10 DAI and 15 DAI in fresh rice hull + soil carrier. In slag + soil carrier, the highest population of 3.21 x 10^6 cfu g^{-1} (log number of cells, 6.51) was observed at 45 DAI. The log number of bacterial cells in slag + soil and fresh bagasse + soil carriers range from 5.48-6.51 and 5.43-6.70, respectively. The highest population of 5.0 x

10^6 cfu g^{-1} (log number of cells, 6.70) was obtained in fresh bagasse + soil carrier at 150 DAI with a final population of 4.6 x 10^6 cfu g^{-1} at 180 DAI (log number of cells, 6.66). Based on the present study, slag + soil and fresh bagasse + soil carriers are potential SSB carrier due to their good survival rates.

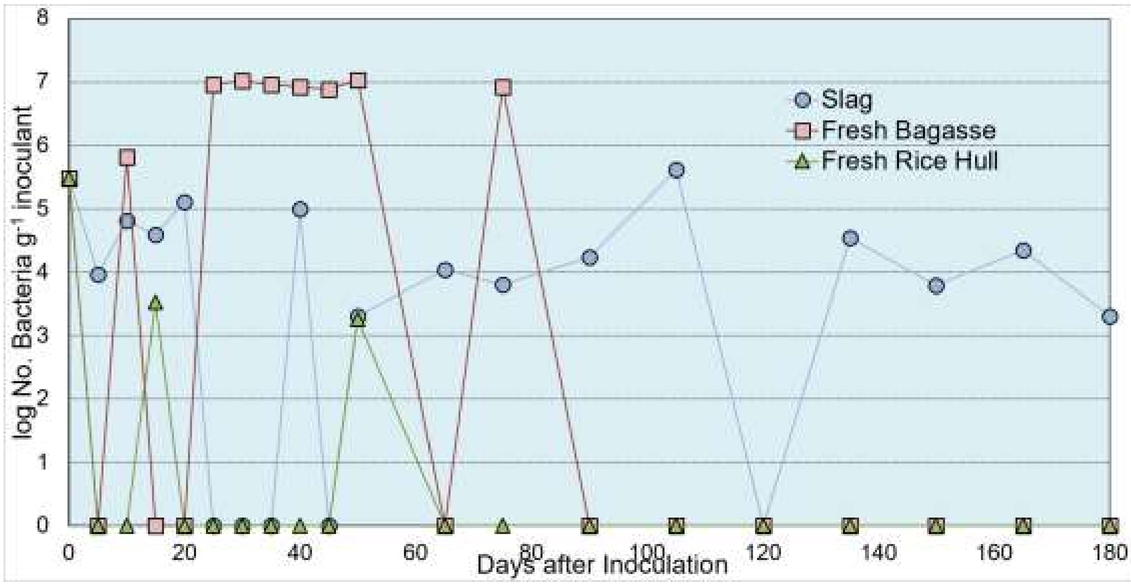


Figure 4.3. Survival of silicate-solubilizing bacteria (SSB) on slag, fresh bagasse, and fresh rice hull carriers at 180 days after inoculation.

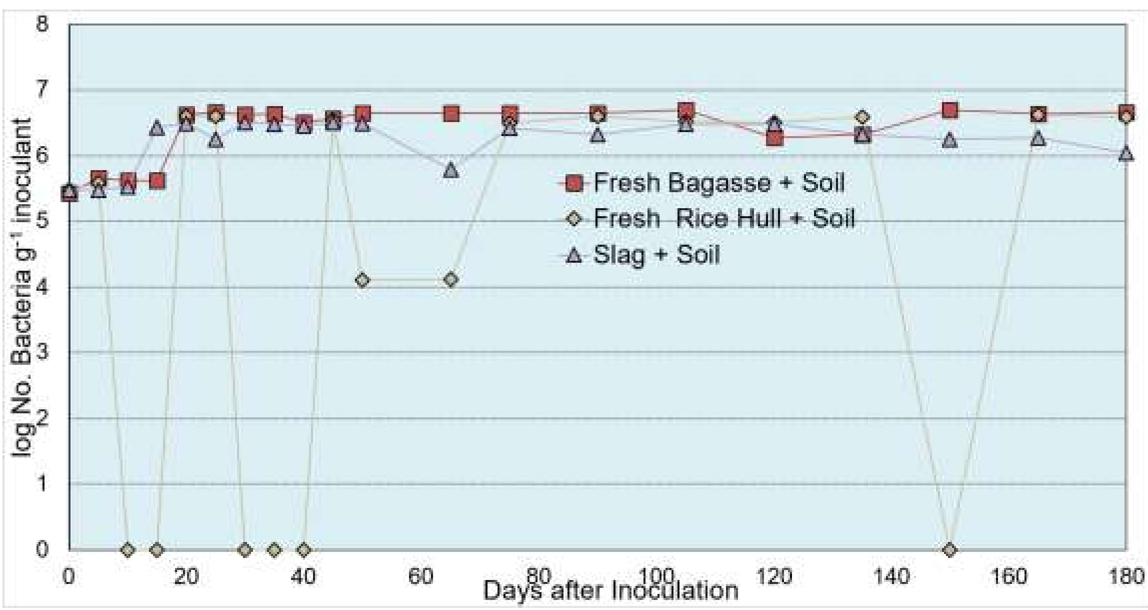


Figure 4.4. Survival of silicate-solubilizing bacteria (SSB) on slag + soil, fresh bagasse + soil, and fresh rice hull + soil carriers at 180 days after inoculation.

Soil is the common component of the three given inoculant carriers: slag + soil, fresh bagasse + soil, and fresh rice hull + soil. Another factor affecting the survival and growth of microorganisms is soil pH. The pH of the soil used in this experiment is 6.6. Most of the microorganisms are adapted at the pH range of 5.0-9.0 which is the pH of most natural environments. Enzymes work best at pH close to that of the environment. On the other hand, acidic conditions present a particularly stressful situation to the microbial cell (Tate III, 2000). Hence, it is also imperative to understand the effect of soil pH on soil microbial community development and function.

Figure 4.5 shows the number of viable cells of SSB as a function of time in fresh bagasse, burned bagasse + soil, and fresh bagasse + soil carriers. In general, there was rapid multiplication of bacteria in all the carriers starting 10 DAI. Although there were differential population densities of SSB in the three carrier materials, the organism showed a good survival rate up to 180 days except for fresh bagasse. The maximum cell population of SSB was supported by burned bagasse + soil (4.9 X 10^6 cfu g^{-1} --180 DAI), followed by fresh bagasse + soil (3.9 X 10^6 cfu g^{-1} --150 DAI). Except in the case of fresh bagasse, the number of cells started to decrease after 50 DAI. According to Smith (1992), the three main characteristics of a good carrier are the following: high nutrient content, high water holding capacity and good aeration properties. In the present study, the description of different carrier materials is shown Tables 4.1-4.2. Burned bagasse + soil and fresh bagasse + soil have 52% and 40% moisture content (MC), respectively. Amount of culture broth added to each carrier material was adjusted based on the % MC or maximum water holding capacity.

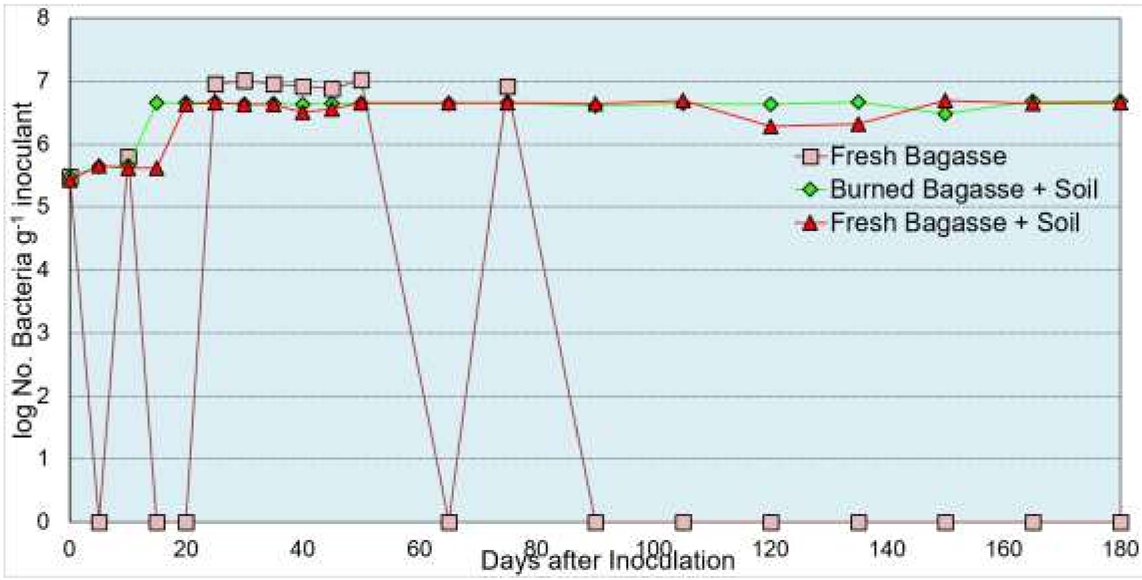


Figure 4.5. Survival of silicate-solubilizing bacteria (SSB) on fresh bagasse, burned bagasse + soil and fresh bagasse + soil carriers at 180 days after inoculation.

Bagasse is one of the agricultural waste products in sugarcane (*Saccharum spp*.) production. Sugarcane-bagasse is dried lignocellulosic remains of sugarcane left after extraction of the juice for the manufacture of sugar. In Khavazi et al. (2007) study, sugarcane bagasse and perlite were tested as carriers for *Bradyrhizobium japonicum strain* CB1809. Similarly, sugarcane-bagasse, sawdust, and rice husk are being used in India as potential inoculant carriers specifically for *Burkholderia* sp. Pandey and Maheshwari (2007) evaluated different low-cost carriers for the formulation of an effective bio-inoculant with *Burkholderia* sp. Bagasse was also a good source of P and K. Whey and bagasse have been reported to be acceptable C and N sources for rhizobial culture. Carrier materials with better C:N ratio and inorganic content are good conditioners of soil and they also support the growth of bacterial cells (Pandey and Maheshwari, 2007). In the present study, the C:N ratio of fresh bagasse and burned bagasse were 137 and 35.48, respectively (See Table 2).

However, Muniruzzaman and Khan (1992) stated in their study that with sugarcane bagasse there was also a difference in survival rate of bacteria due to strain variability in survival characteristics. In the study of Doni et al. (2014), *Trichoderma* sp. showed a good mycelium growth using bagasse as the carrier. *Trichoderma* sp. Sugarcane bagasse and wheat (*Triticum aestivum*) bran can also be used as carrier for N_2-fixing cyanobacteria such as *N. commune* (Alla and Issa, 1994).

Figure 4.6 shows the survival of SSB on fresh rice hull, burned rice hull + soil and fresh rice hull + soil carriers at 180 DAI. The initial population of SSB in burned rice hull + soil and fresh rice hull + soil carriers of 3.0 x 10^5 cfu g^{-1} increased to 3.9 x 10^5 cfu g^{-1} and 3.9 x 10^5 cfu g^{-1}, respectively 5 DAI. However, there was a drastic decrease in SSB population at 5 and 10 DAI in fresh rice hull carrier.

In fresh rice hull carrier, the highest population of 3.4 x 10^3 cfu g^{-1} (log number of cells, 3.53) was observed at 15 DAI. The log number of bacterial cells in burned rice hull + soil and fresh rice hull + soil carriers ranged from 5.48-6.66 and 5.43-6.63, respectively. The highest population of 4.5 x 10^6 cfu g^{-1} (log number of cells, 6.66) was obtained in burned rice hull + soil carrier at 90 DAI with a final population of 4.1 x 10^6 cfu g^{-1} at 180 DAI (log number of cells, 6.61). Overall, the burned rice hull + soil and fresh rice hull + soil carriers are good candidates for SSB carrier. The SSB showed a good survival rate up to 180 days except for fresh rice hull.

Rice husk (hull) is a fibrous, non-digestible by-product of rice production that makes up about 20% of the weight of rough rice (Hashim et al., 1996). Researchers were looking into the potential application of rice hull, an agricultural by-product, as an inoculant carrier due to its low cost. It is also valued for its high amorphous silica content of potential nutritional benefit to plants (Khatri et al., 1973; Brockwell and Bottomley, 1995; Yardin et al., 2000; Ben Rebah et al., 2007; Khavazi et al., 2007). In the study of Hafeez et al. (1989), they found that rice hull is a marginally effective inoculant carrier for *Bradyrhizobium*. However, Nguyen et al. (2003) found a statistically significant increase in rice yield using rice hull + clay soil as a carrier for multi-strain biofertilizer. The formulation consisted of the following: clay soil (50%), rice husks (25%), sugar (1%), plus water and broth culture (24%).

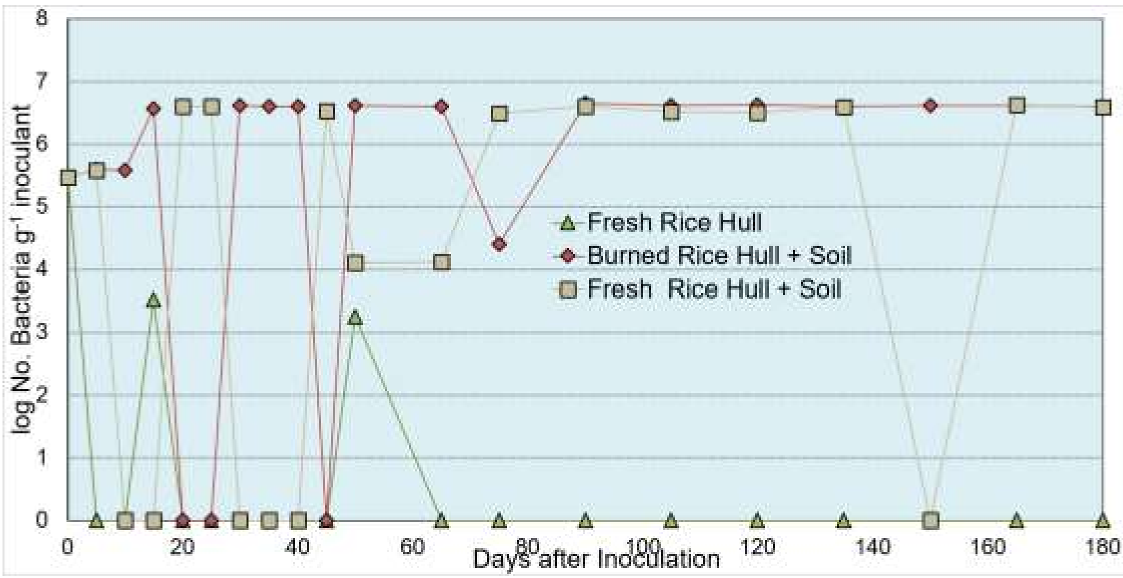


Figure 4.6. Survival of silicate-solubilizing bacteria (SSB) on fresh rice hull, burned rice hull + soil and fresh rice hull + soil carriers at 180 days after inoculation.

Figure 4.7 shows the survival of SSB on slag and slag + soil carriers. The initial population of SSB in slag of 3.0 x 10^5 cfu g^{-1} decreased to 9.1 x 10^3 cfu g^{-1} (log number of cells, 3.96) 5 DAI followed by an unstable survival rate until the end of incubation period of 180 days. The maximum cell population of SSB in slag at 4.1 X 10^5 cfu g^{-1} (log number of cells, 5.62) was observed at 105 DAI. In slag + soil carrier, the highest population of 3.1 x 10^6 cfu g^{-1} (log number of cells, 6.51) was observed at 45 DAI with a final population of 1.0 x 10^6 cfu g^{-1} at 180 DAI (log number of cells, 6.01). This result suggests that slag + soil carrier is better than the slag as a carrier. The SSB showed a good survival rate up to 180 days in slag + soil carrier.

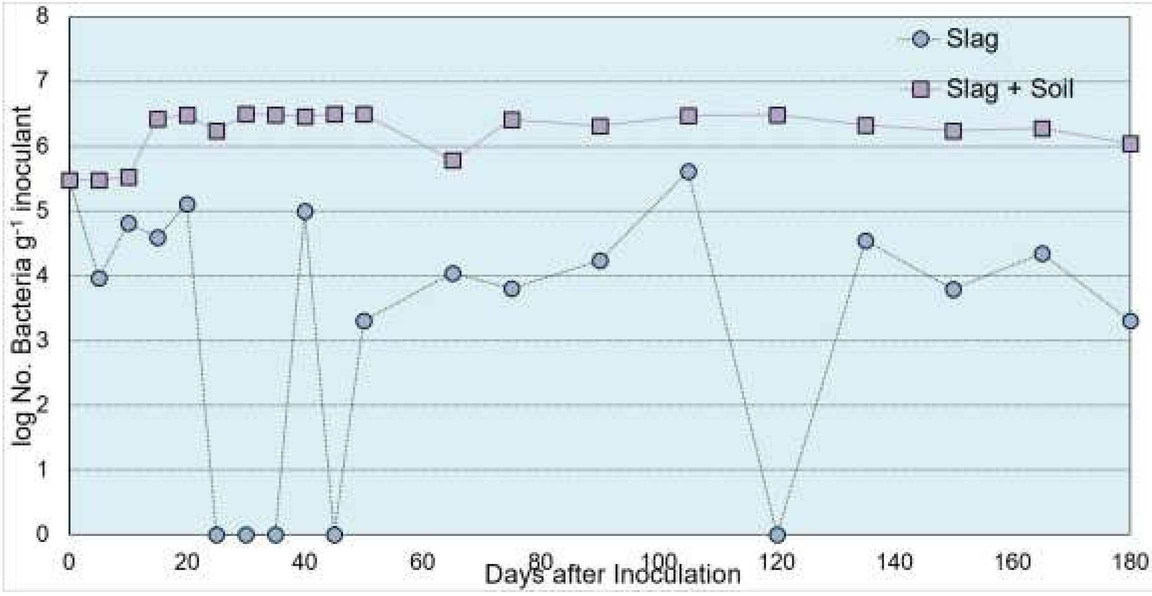


Figure 4.7. Survival of silicate-solubilizing bacteria (SSB) on slag alone and slag + soil carriers.

Figure 4.8 shows the survival of SSB on eight different carriers: slag, fresh bagasse, fresh rice hull, slag + soil, burned bagasse + soil, burned rice hull + soil, fresh bagasse + soil, and fresh rice hull + soil. Based on the present study, SSB showed a good survival rate up to 180 days in slag + soil, burned bagasse, bagasse + soil, and burned rice hull + soil carriers. Hence, we can say that these carriers are potential candidates as inoculant carriers for the SSB.

A variety of materials used as carriers has been shown to improve the survival and biological effectiveness of inoculants by protecting bacteria from biotic and abiotic stresses (Van Veen et al., 1997). Suitable carriers should be cheap, easy-to-use, and accessible. Also, the carrier must permit gas exchange, particularly oxygen, and has high water holding capacity as well (Bashan, 1998; Ben Rebah et al., 2002). According to Somasegaran and Hoben (1994), a good carrier material must be non-toxic either to the bacterial inoculants or to the plant itself. Furthermore, Stephens and Rask (2000) and Ferreira and Castro (2005) stated that carriers should have near neutral or readily adjustable pH, be abundant locally at a reasonable cost and able to sterilize. Also, it should be able to support microorganism growth and survival and easily release functional microorganisms into the soil (Wang et al., 2015). These properties only indicate the potential for a good carrier, while final selection of carrier must be based on microbial multiplication and survival during storage.

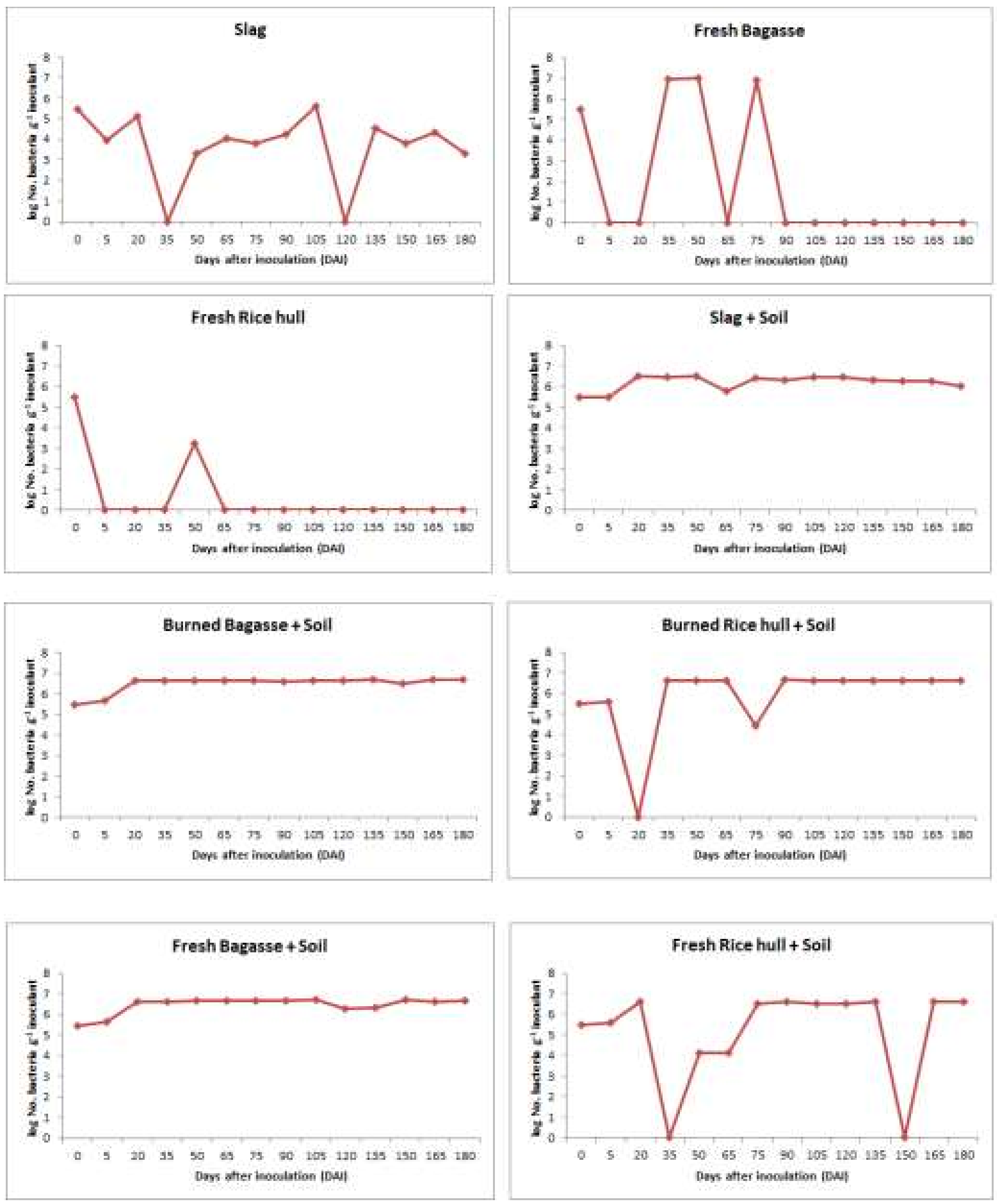


Figure 4.8. Survival of silicate-solubilizing bacteria (SSB) on different carriers: slag, fresh bagasse, fresh rice hull, slag + soil, burned bagasse + soil, burned rice hull + soil, fresh bagasse + soil, and fresh rice hull + soil.

4.3.2. Construction of GFP-tagged SSB

The plasmid pBB2rpoDGFP1 was successfully transformed into WinnsB-6 isolate (Figure 4.9a). The bacterial cells with plasmid were showing green fluorescence when the bacterial culture on the microscopic glass slide was observed under a fluorescence microscope, confirming successful transformation of the green fluorescence protein (GFP) containing plasmid into the recipient strains (Figure 4.9b).

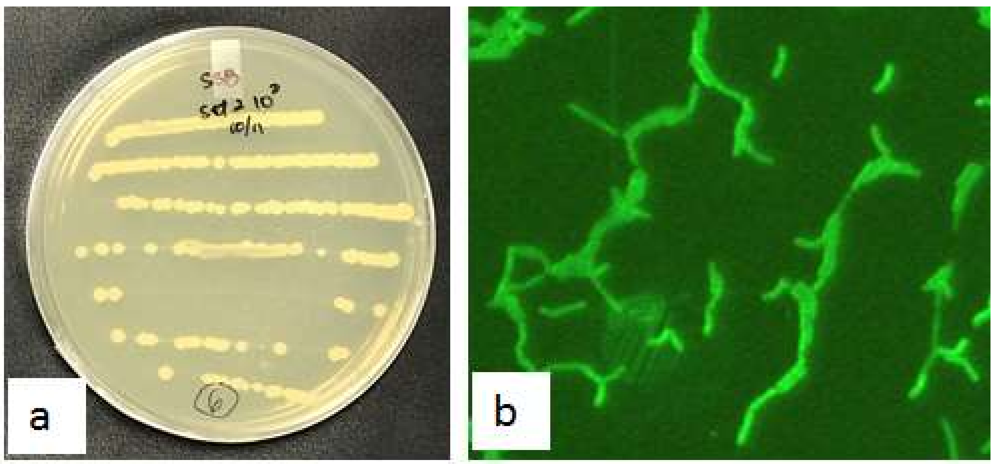


Figure 4.9. Silicate-solubilizing bacteria (SSB) transformant **(a)** and visualization of GFP-tagged SSB under a fluorescence microscope (Leica DM6B Microscope) **(b)**. Bacterial cells on a glass slide show a green fluorescence.

4.3.3. Colonization and Visualization of SSB in Rice Plants

Plants were sampled at 14 days after bacterial inoculation on the rice seeds. Under laboratory conditions, Confocal Laser Scanning Microscope viewing revealed the successful GFP tagging of WinnsB-6 isolate visible inside the two-week old rice root seedlings grown from inoculated seed (Figure 10 c and d). On the other hand, no fluorescing cells were detected on the root surface and inside of the roots of the control (non-inoculated) plants when observed under CLSM indicating the absence of GFP-fluorescent signals (Figures 4.10a and 4.10b). The bright green dots (bacteria) inside the rice roots indicate the cells of the WinnsB-6 isolate that colonized the rice roots (Figure 4.10c and 4.20d). However, the bacterial bright green dots were not detected on the root surface of the inoculated rice plants when observed under CLSM.

When rice seedlings treated with GFP-labeled WinnsB-6 isolate was observed under CLSM, bacterial cells with bright green fluorescence were detected only inside the rice root tissues but not on the surface. This indicates the ability of SSB to colonize the root tissues of the two-week old rice seedlings and demonstrates the ability of WinnsB-6 isolate to survive when used as a seed treatment, which is a very practical and efficient application method of potential bio-inoculant to the field in the future.

The bright green dots/fluorescence seemed to be few in the rice seedlings. Although green fluorescence was detected on the GFP-tagged WinnsB-6 during the transformation, there were probably few surviving GFP-tagged WinnsB-6 at 14 days after seeds inoculation. The growth and survival of bacteria may have influenced the pH and other conditions in the culture solution of the hydroponic system used, thus limited the bacterial survival. In the study of Poonguzhali et al. (2008), high background fluorescence and lack of bacterial aggregation prevented easy visualization of CBMB120-gfp29 in rice. No fluorescing cells were observed in control roots of rice, while sparsely distributed single rod or circular shaped cells colonizing the rhizoplane were observed in CBMB120-gfp29 treated rice plants (Poonguzhali et al., 2008). The bacterial strains tagged with GFP have been utilized in many studies to study the colonization potential of *Methylobacterium suomiense*, *Bacillus* sp., *Rhizobium* sp., and *Burkholderia* sp. in rice plants (Liu et al., 2006; Poonguzhali et al., 2008; Singh et al., 2009). Most of the plant-growth-promoting bacteria (PGPB) are applied through seed coating or soil source. Rhizosphere competence is considered as a crucial factor in determining the success of plant-growth promotion by PGPB. Hence in the present study, understanding the colonization pattern of potential SSB (WinnsB-6) and its survival is a critical prerequisite when introduced through a seed source.

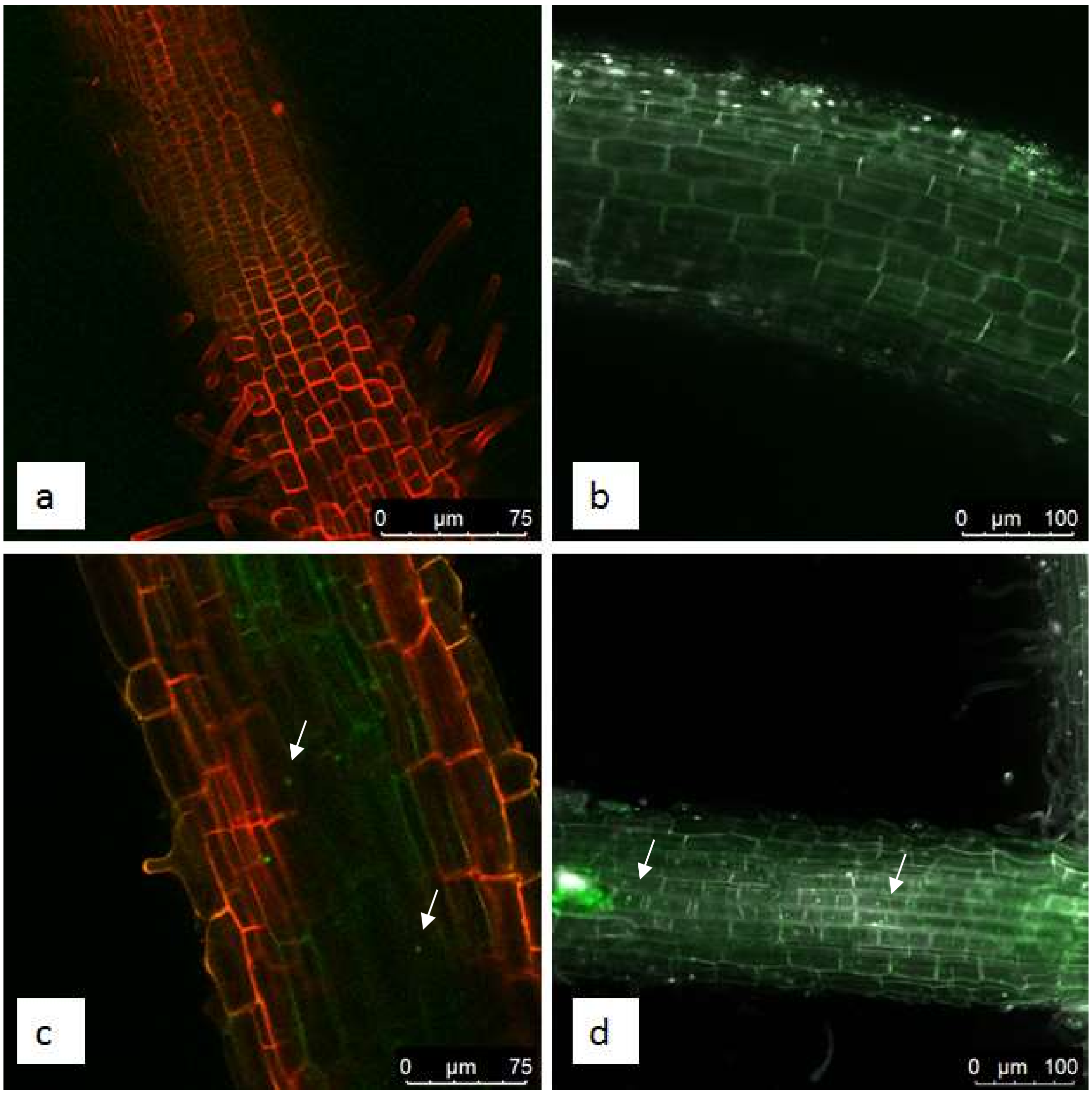


Figure 4.10. Visualization of GFP-tagged bacteria in rice plants using different background colors. Bright green dots were not observed on the rice roots, indicating the absence of GFP-tagged WinnsB-6 **(a and b, control-uninoculated)**. Bright green dots were observed inside the rice roots, indicating the presence of GFP-tagged WinnsB-6 **(c and d, inoculated)**. The presence of GFP-tagged WinnsB-6 was confirmed under a confocal laser scanning microscope (CLSM). Root samples were stained with propidium iodide to stain the cell walls.

Green fluorescence protein provides a convenient tool to assess the survival rates of potential PGPB or bio-inoculant when applied to the crops. Survival rates of bacteria are very important as it will affect and influence the crop growth especially on its critical stages. WinnsB-6 isolate should be further studied starting from the time of seed treatment to rice plant maturity under greenhouse and field conditions to evaluate the ability of bacteria to survive under different conditions.

4.4. Conclusions

Survival of SSB was evaluated on eight different carriers. The components of the carriers were soil plus bagasse, rice hull and slag. These agricultural and industrial waste products have potential application as an inoculant carrier due to its low cost and good source of other nutrients. The highest population of 5.0 x 10^6 cfu g^{-1} (log number of cells, 6.70) was obtained in bagasse + soil carrier at 150 days after inoculation with a final population of 4.6 x 10^6 cfu g^{-1} at 180 days after inoculation (log number of cells, 6.66). One of the factors affecting the survival and growth of microorganisms is soil pH. The pH of the soil used in this experiment is 6.6. Most of the microorganisms are adapted at the pH range of 5.0-9.0 which is the pH of most natural environments. Enzymes work best at pH close to that of the environment. One possible reason for higher levels of bacterial population at maturity is their ability to produce growth hormone indole3-acetic acid which increases the exudation from the cells. The utilization of what seemed to be a waste product is a timely topic of research. The present study has shown that these agricultural wastes could not only serve as excellent carriers for the preparation of bacterial inoculants but that their use would also result in an economic utilization of these wastes and pollution control.

The results on fluorescent microscopy showed that SSB can colonize the root tissues of the two-week old rice seedlings indicating its ability to survive when used as a seed treatment, which is a very practical and efficient application method of potential bio-inoculant to the field in the future. Understanding the colonization of potential SSB and its survival is a very important consideration in making an inoculant formulation. Green fluorescence protein provides a convenient tool to assess the survival rates of potential plant growth-promoting bacteria (PGPB) or bio-inoculant when applied to the crops. Survival rates of bacteria are very important as it will affect and influence the crop growth especially on its critical stages.

Chapter 5. Conclusions

Silicate-solubilizing bacteria (SSB) were isolated from Louisiana soils and proved to produce multiple plant growth-promoting compounds such as phosphatase, nitrogenase, ACC deaminase, and indole-3-acetic acid enzyme. Potential SSB were identified into four genera: *Aeromonas, Bacillus, Enterobacter* and *Pseudomonas*. In the greenhouse, the differences in agronomic variables and silicon (Si) nutrition of rice were evident and consistent between the soil types. While Si addition did not result in significant grain yield increase, there was a significant improvement observed on rice Si uptake. The survival test confirmed the presence of SSB in the different carriers. Thus the improvement on straw Si content of rice seeds could be associated with the use of SSB-inoculated carriers. Even so, this did not result in improvement on rice biomass and grain yield. The lack of yield response to Si addition was partly explained by the high initial soil Si availability and if not completely absent, minimal occurrence of growth limiting factors during the growing period. The semi-quantitative evaluation of silica bodies distribution on leaf surface of rice (treated with wollastonite and SSB using different carriers) via SEM-EDX differed between soil types. Greater distribution of silica bodies was observed in rice planted in Commerce silt loam.

In the laboratory, the highest population of SSB, 5.0 x 10^6 cfu g^{-1} (log number of cells, 6.70), was obtained in bagasse + soil carrier at 150 days after inoculation with a final population of 4.6 x 10^6 cfu g^{-1} at 180 days after inoculation (log number of cells, 6.66). One of the factors affecting the survival and growth of microorganisms is soil pH. The pH of the soil used in this experiment is 6.6. Most of the microorganisms are adapted at the pH range of 5.0-9.0 which is the pH of most natural environments. The present study has shown that these agricultural wastes could not only serve as excellent carriers for the preparation of bacterial inoculants but their use would also result in an economic utilization of these wastes.

Fluorescent microscopy analysis proved the ability of GFP-SSB to colonize the root tissues of the two-week old rice seedlings indicating its ability to survive when used as a seed treatment, which is a very practical and efficient application method of potential bioinoculant to the field in the future. Green fluorescence protein provides a convenient tool to assess the survival rates of potential plant growth-promoting bacteria (PGPB) or bioinoculant when applied to the crops. In this study, field assessment is needed to provide more information on the effect of biotic and abiotic stresses on soil types, silicon addition, and SSB grown on different carriers on rice productivity.

Overall, the outcomes of this research suggest that SSB inoculation is a "ready" approach in improving Si availability to crops but still the benefits of this potential technology were not fully realized. With notable amount of research work was done on Si fertilization and soil testing, little has been done on the role of microorganisms in plant Si nutrition. Further evaluation on the effectiveness of this SSB is needed. For future research, the following factors could be improved: concentration of SSB and method and time of application. Environmental concerns and rising cost of chemical fertilizers are some major concerns in crop production. Hence, alternative technologies like utilizing beneficial microorganisms like SSB should be implemented. This would offer the industry a practical, innovative, and ecologically-smart crop care solution, not to mention its huge potential as a commercial product.

Appendix. Supplementary Materials for Chapters 2 and 3.

Chapter 2

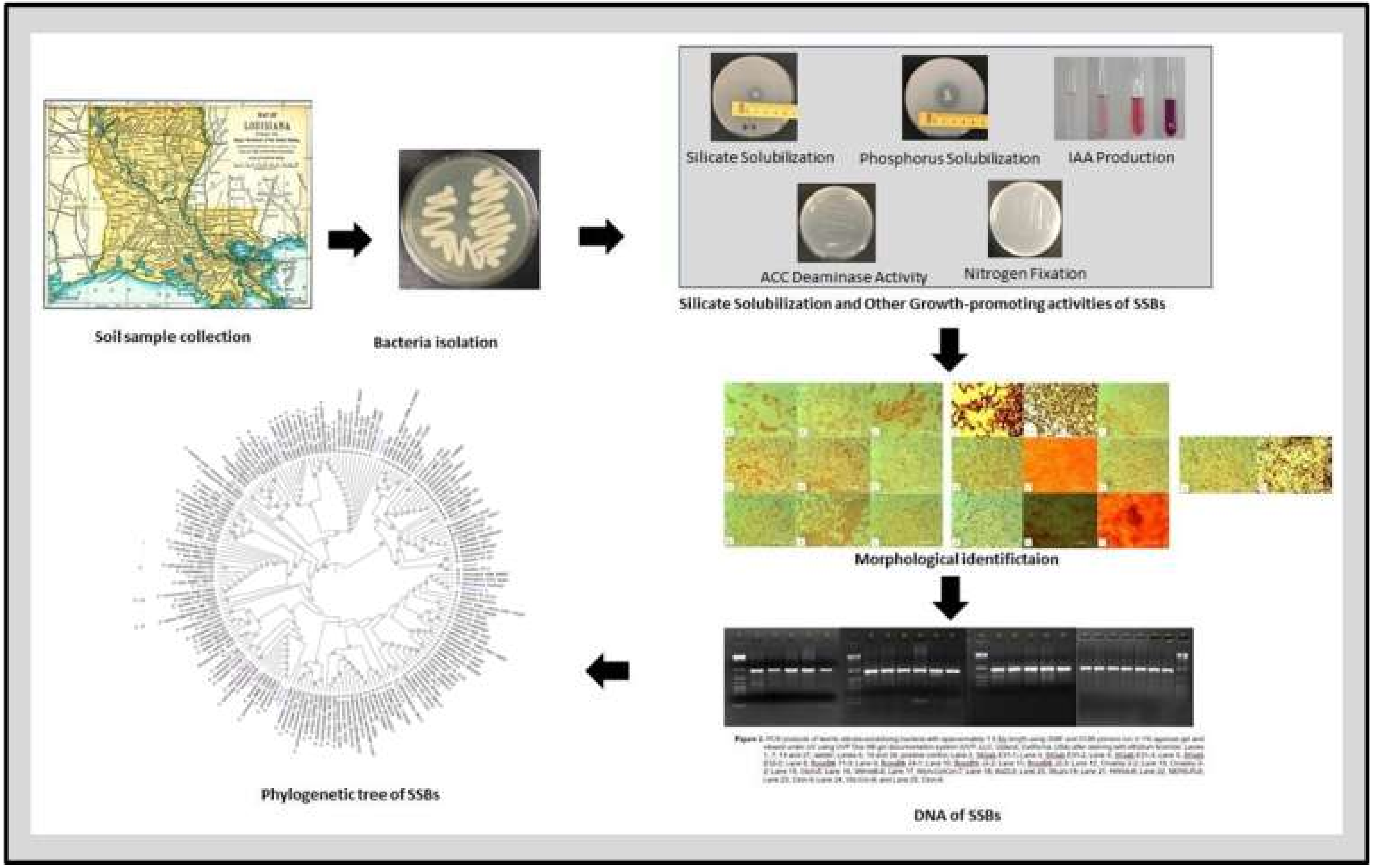


Figure A.1. Flowchart of the study.

Chapter 3

Table A.1. Description of the different carrier materials and the amount of inoculum used in this study.

Treatment	Treatment Code	Description	Moisture content (%)	Inoculum added (ml100 ml^{-1} broth)
1	Slag	100% slag	16.00	4.12
2	Fresh bagasse	100% fresh rice hull	63.00	1.05
3	Fresh rice hull	100% fresh bagasse	66.00	1.00

Table A.2. Results on analysis of variance for shoot essential nutrient content at harvest in 2019.

Sources of Variation	B	Ca	Cu	Fe	Mg	Mn	Mo	Ni	P	K
Soil (S)	0.1984	0.1426	0.5222	**0.0008**	0.1221	**<0.0001**	**<0.0001**	**0.0006**	**<0.0001**	0.0650
Si Addition (Si)	0.1887	0.0685	0.4291	0.2512	**0.0116**	**0.0143**	0.1124	0.0682	0.6332	0.5408
Carrier (C)	0.9332	0.7292	0.9006	0.5551	0.5229	0.1154	0.3696	0.6684	0.6588	0.9947
S x Si	0.1847	0.2550	0.2225	0.3608	0.1039	**0.0199**	0.0917	**0.0051**	0.4413	0.2506
S x C	0.5147	0.5660	0.8146	0.5165	0.6882	0.1055	0.5985	0.9715	0.9958	0.8766
Si x C	0.9317	0.5745	0.2794	0.5545	0.5797	0.8220	0.8761	0.6132	0.8126	0.3319
S x Si x C	0.8856	0.4835	0.4645	0.2312	0.4523	0.8450	0.9257	0.7051	0.3925	0.3116

Table A.3. Results on analysis of variance for root essential nutrient content at harvest in 2019.

Sources of Variation	B	Ca	Cu	Fe	Mg	Mn	Mo	Ni	P	K
Soil (S)	0.1682	**<0.0001**	0.5743	**0.0003**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0009**	0.8179
Si Addition (Si)	0.5122	0.4063	0.2095	0.5225	0.1744	0.8168	0.6307	**0.0239**	0.3383	0.9418
Carrier (C)	0.1148	0.8160	0.8873	0.3937	0.5515	0.3528	0.8250	0.2460	0.3881	0.1341
S x Si	0.9411	0.9763	0.3506	0.8892	0.6853	0.8041	0.8583	0.1989	0.6858	0.5771
S x C	0.2314	0.9950	0.9835	0.7286	0.5366	0.4532	0.8509	0.2076	0.5090	0.5391
Si x C	0.4942	0.8242	0.6030	0.5820	0.1851	0.1884	0.7956	0.2768	0.6096	0.8064
S x Si x C	0.4609	0.7905	0.5848	0.1826	0.4031	0.1911	0.1796	0.3655	0.2261	0.1325

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Vita

Jayvee A. Cruz was born in Bulacan, Philippines in January of 1986. She is presently a Senior Science Research Specialist at the Philippine Rice Research Institute (PRRI). She obtained her B.S. Agriculture major in Soil Science in 2009 and M.S. Soil Science minor in Molecular Biology and Biotechnology in 2013, from the University of the Philippines, Los Baños (UPLB). In May of 2018, she got accepted in the Ph.D. Program in the School of Plant, Environmental, and Soil Sciences at Louisiana State University under the mentorship of Dr. Brenda S. Tubana. Her research revolved around the use of plant growth-promoting microorganisms in improving crop productivity. She focused on identification and profiling of silicate-solubilizing bacteria in agricultural soils in Louisiana. Over the years, Ms. Cruz' in-depth work on Soil Fertility-Soil Microbiology has broken new grounds in rice soil science. At the macro level, her research results may prove pivotal in suppressing problems on nutrient deficiency (specifically silicon) in agricultural soils while lessening chemical fertilizer use hence, offering the industry a practical, innovative, and ecologically-smart crop care solution, not to mention its huge potential as a commercial product.